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(57) Abstract

The bacterial serine protease, subtilisin BPN', has been mutated so that it will efficiently and selectively cleave substrates containing basic residues. Combination mutants, where Asn 62 was changed to Asp, Gly 166 was changed to Asp (N62D/G166D), and optionally Tyr 104 was changed to Asp had a larger than additive shift in specificity toward substrates containing basic residues. Suitable substrates of the subtilisin variants were revealed by sorting a library of phage particles (substrate phage) containing five contiguous randomized residues. This method identified a particularly good substrate, Asn-Leu-Met-Arg-Lys- (SEQ ID NO: 35), that was selectively cleaved in the context of a fusion protein by the N62D/G166D subtilisin variant. A particularly good substrate for N62D/G166D/Y104D would be Asn-Arg-Met-Arg-Lys- (SEQ ID NO: 76). Accordingly, these subtilisin variants are useful for cleaving fusion proteins with basic substrate linkers and processing hormones or other proteins *in vitro* or *in vivo* that contain basic cleavage sites.

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SUBTILISIN VARIANTS CAPABLE OF CLEAVING SUBSTRATES CONTAINING BASIC RESIDUES

FIELD OF THE INVENTION

This invention relates to subtilisin variants having altered specificity from wild-type subtilisins. Specifically, the subtilisin variants are modified so that they efficiently and selectively cleave substrates containing basic residues. The invention further relates to the DNA encoding these novel polypeptides, as well as the recombinant materials and methods for producing these subtilisin variants. In a particular aspect, the present invention provides for processes for cleaving protein substrates containing basic residues.

BACKGROUND OF THE INVENTION

Site-specific proteolysis is one of the most common forms of post-translational modifications of proteins (for review see Neurath, H. (1989) Trends Biochem. Sci., 14:268). In addition, proteolysis of fusion proteins *in vitro* is an important research and commercial tool (for reviews see Uhlen, M. and Moks, T. (1990) Methods Enzymol., 185:129-143; Carter, P. (1990) in Protein Purification: From Molecular Mechanisms to Large-Scale Processes, M.R. Landisch, R.C. Wilson, C.D. Painton, S.E. Builder, Eds. (ACS Symposium Series 427, American Chemical Society, Washington, D.C.), Chap. 13, p.181-193; and Nilsson, B. et al. (1992) Current Opin. Struct. Biol., 2:569). Expressing a protein of interest as a fusion protein facilitates purification when the fusion contains an affinity domain such as glutathione-S-transferase, Protein A or a poly-histidine tail. The fusion domain can also facilitate high level expression and/or secretion.

To liberate the protein product from the fusion domain requires selective and efficient cleavage of the fusion protein. Both chemical and enzymatic methods have been proposed (see references above). Enzymatic methods are generally preferred as they tend to be more specific and can be performed under mild conditions that avoid denaturation or unwanted chemical side-reactions. A number of natural and even designed enzymes have been applied for site-specific proteolysis. Although some are generally more useful than others (Forsberg, G., Baastrup, B., Rondahl, H., Holmgren, E., Pohl, G., Hartmanis, M. and Lake, M. (1992) J. Prot. Chem., 11:201-211), no one is applicable to every situation given the sequence requirements of the fusion protein junction and the possible existence of protease sequences within the desired protein product. Thus, an expanded array of sequence specific proteases, analogous to restriction endonucleases, would make site-specific proteolysis a more widely used method for processing fusion proteins or generating protein/peptide fragments either *in vitro* or *in vivo*.

The processing of prohormones by the KEX2-related family of serine endoproteases illustrates one of the most precise proteolytic events found in nature (for reviews see Steiner, D. F.,Smeekens, S. P., Ohagi, S. and Chan, S. J. (1992)J. Biol. Chem., 267, 23435-23438 and Smeekens, S. P. (1993) Bio/Technology 11, 182-186). This family of proteases, that includes the yeast KEX2 and the mammalian PC2, PC3 and furin enzymes, are homologous to the bacterial serine protease subtilisin (Kraut, J. (1977) Annu. Rev. Biochem.., 46:331-358). Subtilisin has a broad substrate specificity that reflects its role as a scavenger protease. In contrast, these eukaryotic enzymes are very specific for cleaving substrates containing two basic residues and thus well-suited for site-specific proteolysis.

All of these eucaryotic enzymes strongly require Arg at the P1 position, and either Arg, Lys or Pro at the P2 position of peptide substrates. The prohormone convertases from higher eukaryotes such as furin, PC2, and PC3 also have an absolute requirement for Arg at the P4 position (Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J. and Thomas, G. (1990) J. Cell. Biol. 111, 2851; Wise, R. J., Baar, P. J., Wong, P. A., Kiefer, M. C., Brake, A. J., and Kaufman, R. J. (1990) Proc. Natl. Acad. Sci. USA 87, 9378-9382.; Hosaka, M., Nagahama, M., Kim, W.-S., Watanabe, T., Hatsuzakawa, K., Ikemizu, J., Murakami, K., and Nakayama, K. (1991) J. Biol. Chem. 266, 12127-12130.; Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994) Protein Science 3, 1197-1205).

Despite the very narrow specificity of the pro-hormone processing enzymes, in some cases they are capable of rapid cleavage of target sequences. For example, the k_{cat}/Km ratio for KEX2 to cleave a good substrate (e.g. acetyl-pMYRK-MCA) is $1.1x10^7$ $M^{-1}s^{-1}$ (Brenner, C., and Fuller, R.S. (1992) Proc. Natl. Acad. Sci. USA , 89:922-926) compared to $3x10^5$ for subtilisin cleaving a good substrate (e.g. suc-AAPF-pNA) (Estell, D. A., Graycar, T. P., Miller, J. V., Powers, D. B., Burnier, J. P., Ng, P. G. and Wells, J.A. (1986) Science, 233:659-663).

However, the eukaryotic proteases are expressed in small amounts (Bravo, D. B., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) *J. Biol. Chem.*, 269:25830-25837 and Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994) *Protein Science* , 3:1197-1205) making them impractical to apply presently to processing of fusion proteins *in vitro*. Subtilisin BPN' however, can be expressed in large amounts (Wells, J.A., Ferrari, E., Henner, D.J., Estell, D.A. and Chen, E.Y. (1983) *Nucl. Acids Res.*, 11:7911-7929)

Extensive protein engineering studies of subtilisin, and especially subtilisin BPN', have identified several residues in the S1 and S2 active site of the enzyme where amino acid substitutions lead to large changes in substrate specificity (Wells, J. A., and Estell, D.A., (1988) Trends Biochem. Sci., 13:291-297; Carter, P., et al., (1989) PROTEINS:Structure, Function, and Genetics, 6:240-248). X-ray crystal structures of subtilisin containing bound transition state analogues (Wright, C. S., Alden, R. A. and Kraut, J. (1969) *Nature* , 221:235-242; McPhalen, C.A. and James, N.G. (1988) Biochemistry, 27:6582-6598; Bode, W., Papamokos, E., Musil, D., Seemueller, U. and Fritz, M. (1986) EMBO J., 5:813-818; and Bott, R., Ultsch, M., Kossiakoff, A., Graycar, T., Katz, B. and Power, S. (1988) J. Biol. Chem., 263:7895-7906) can be used to locate active site residues that are in close proximity to side chains at key positions in substrate peptides (Wells, J.A., (1987) Proc. Natl. Acad. Sci. USA 84:1219-1223). Consideration of electrostatic interactions between charged peptide substrates and subtilisin can be used to tailor the substrate binding cleft of the subtilisin BPN' to favor complementary charged substrates (Wells, J.A., et al., (1987) Proc. Natl. Acad. Sci., USA, 84:1219-1223). Previous work has shown that replacement of residues at position 156 and 166 in the S1 binding site of subtilisin BPN' with various charged residues leads to improved specificity for complementary charged substrates.

A substantial amount of protein engineering has been applied to the specificity determinants of the S4 subsite of subtilisin BPN' in efforts to alter specificity for P4 substrates (Eder, J., Rheinnecker, M., and Fersht, A. R. (1993) FEBS Lett 335, 349-352; Rheinnecker, M., Baker, G., Eder, J., and Fersht, A. R. (1993) Biochemistry 32, 1199-1203; Rheinnecker, M., Eder, J.,Pandey, P.S., and Fersht, A. R. (1994) Biochemistry 33,

221-225). However, the mutations introduced consisted entirely of hydrophobic substitutions, thus preserving the overall hydrophobic substrate preference in the site.

Previous attempts to introduce, remove or reverse charge specificity in enzyme active sites have been met with considerable difficulty. This has generally been attributed to a lack of stabilization of the introduced charge or enzyme-substrate ion pair complex by the wild-type enzyme environment (Hwang, J.K. and Warshel, A. (1988) Nature , 334:270-272). For example, Stennicke *et. al* (Stennicke, H.R.; Ujje, H.M.; Christensen, U.; Remington, S.J.; and Breddam (1994) Prot. Eng. 7:911-916) made acidic (D/E) mutations at five residues in the P1' binding of carboxypeptidase Y in an attempt to change the P1' preference from Phe to Lys/Arg. Only the L272D and L272E mutations were found to alter the specificity in the desired direction, up to 1.5-fold preference in Lys/Arg over Phe, and the others simply resulted in less active enzymes having substrate preferences similar to wild-type. In the case of trypsin, a protease that is highly specific for basic P1 residues, recruitment of chymotrypsin-like (hydrophobic P1) specificity required not only mutations of the ion pair-forming Asp 189 to Ser, but also transplantation of two more distant surface loops from chymotrypsin (Graf, L., Jancso, A., Szilagyi, L., Hegyi, G., Pinter, K., Naray-Szabo, G., Hepp, J., Medzihradszky, K., and Rutter, W. J., Proc. Natl. Acad. Sci. USA (1988) 85:4961-4965 and Hedstrom, L., Szilagyi, L., and Rutter, W. J., *Science* (1992) 255:1249-1253).

In the present work, we have also verified that relatively low specificity is gained by introducing single ion-pairs between enzyme and substrate. However, when two or more choice ionic interactions were simultaneously engineered into subtilisin BPN', the resulting variants had higher specificity for basic residues in each of the subsites due to a non additive effect.

Accordingly, it is an object to produce a subtilisin variant with basic specificity for use in processing pro-proteins made by recombinant techniques.

SUMMARY OF THE INVENTION

The present invention provides for subtilisin variants with altered substrate specificity. Preferred subtilisin variants are highly specific for the efficient cleavage of substrates containing basic residues. The subtilisin variants have a substrate specificity which is substantially different from the substrate specificity of the precursor subtilisin from which the amino acid sequence of the variant is derived. The amino acid sequence of the subtilisin variants are derived by the substitution of one or more amino acids of a precursor subtilisin amino acid sequence.

In a preferred aspect of the present invention, the subtilisin variants of the present invention are specific for the cleavage of protein substrates containing basic amino acid residues at substrate positions P1, P2 and P4. According to this aspect of the present invention subtilisin variants having amino acid substitutions at positions corresponding to amino acid positions 62, 104 and 166 of subtilisin BPN' produced by *Bacillus amyloliquefaciens* are preferred. Accordingly, subtilisin variants are provided wherein amino acids 62, 104 and 166 of subtilisin BPN' are substituted with an acidic amino acids. Preferably the acidic amino acid is Asp or Glu, and most preferably Asp.

Preferred substrates for the subtilisin variants according to this aspect of the present invention contain either Lys (K) or Arg (R) at substrate positions P2 and P1, practically any residue at P3, and preferably either Lys or Arg at P4, and again practically any residue at P5. Thus an exemplary good substrate would contain -Asn-Arg-Met-Arg-Lys- (SEQ ID NO: 76) at -P5-P4-P3-P2-P1- respectively. Additionally, good substrates would not have Pro at P1', P2', or P3' nor would Ile be present at P1'.

According to a second aspect of the present invention the subtilisin variants are capable of cleaving protein substrates having basic residues at positions P1 and P2. According to this aspect of the present invention subtilisin variants having amino acid substitutions at positions corresponding to amino acid positions 62, and 166 of subtilisin BPN' produced by *Bacillus amyloliquefaciens* are preferred. The preferred subtilisin variants having substrate specificity for dibasic substrates have an acidic amino acid residue at residue position 62 of subtilisin naturally produced by *Bacillus amyloliquefaciens*. In a preferred embodiment, the naturally occurring Asn at residue position 62 of subtilisin BPN' is preferably substituted with an acidic amino acid residue such as Glu or Asp, and most preferably Asp. The preferred subtilisin variants, having substrate specificity for substrates having dibasic amino acid residues, additionally have an acidic residue, Asp or Glu, at residue position 166 of subtilisin BPN'. Thus, the subtilisin BPN' variant containing substitution of amino acids 62 and 166 with acidic amino acids Glu or Asp are preferred. In particular, a subtilisin variant having amino acid Asp at positions 62 and 166 is preferred (subtilisin BPN' variant N62D/G166D). The subtilisin variants according to this aspect of the invention may be used to cleave substrates containing dibasic residues such as fusion proteins with dibasic substrate linkers and processing hormones or other proteins (*in vitro* or *in vivo*) that contain dibasic cleavage sites.

Preferred substrates for the subtilisin BPN' variant N62D/G166D contain either Lys (K) or Arg (R) at substrate positions P2 and P1, practically any residue at P3, a non-charged hydrophobic residue at P4, and again practically any residue at P5. Thus an exemplary good substrate would contain -Asn-Leu-Met-Arg-Lys-(SEQ ID NO: 35) at -P5-P4-P3-P2-P1- respectively. Additionally, good substrates would not have Pro at P1', P2', or P3' nor would Ile be present at P1'.

The invention also includes mutant DNA sequences encoding such subtilisin variants. These mutant DNA sequences are derived from a precursor DNA sequence which encodes a naturally occurring or recombinant precursor subtilisin. The mutant DNA sequence is derived by modifying the precursor DNA sequence to encode the substitution(s) of one or more amino acids encoded by the precursor DNA sequence. These recombinant DNA sequences encode mutants having an amino acid sequence which does not exist in nature and a substrate specificity which is substantially different from the substrate specificity of the precursor subtilisin encoded by the precursor DNA sequence.

Further the invention includes expression vectors containing such mutant DNA sequences as well as host cells transformed with such vectors which are capable of expressing the subtilisin variants.

The invention also provides for a process for cleaving a polypeptide such as a fusion protein containing a substrate linker represented by the formula:

P4-P3-P2-P1

wherein P4 is a basic amino acid or a large hydrophobic amino acid such as Leu or Met; P3 is an amino acid selected from the naturally occurring amino acids; P2 is a basic amino acid; and P1 is a basic amino acid. The

process includes the step of subjecting the polypeptide to the subtilisin variants described herein under conditions such that the subtilisin variant cleaves the polypeptide.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of a succinyl-Ala-Ala-Pro-BoroPhe (SEQ ID NO: 69) inhibitor bound to the active site of subtilisin BPN' showing the S2 and S1 binding pocket residues subjected to mutagenesis.

Figure 2. Kinetic analysis of S1 binding site subtilisin mutants versus substrates having variable P1 residues. The kinetic constant k_{cat}/Km was determined from plots of initial rates versus substrate concentration for the tetrapeptide series succinyl-Ala-Ala-Pro-Xaa-pNa (SEQ ID NO: 69), were Xaa was Lys (SEQ ID NO: 58), Arg (SEQ ID NO: 59), Phe (SEQ ID NO: 56), Met (SEQ ID NO: 60) or Gln (SEQ ID NO: 61) (defined to the right of the plot).

Figure 3. Kinetic analysis of S2 binding site subtilisin mutants versus substrates having variable P2 residues. The kinetic constant k_{cat}/Km was determined from plots of initial rates versus substrate concentration for the tetrapeptide series succinyl-Ala-Ala-Xaa-Phe-pNa (SEQ ID NO: 70), were Xaa was Lys(SEQ ID NO: 62), Arg (SEQ ID NO: 64), Ala (SEQ ID NO: 63), Pro (SEQ ID NO: 56), or Asp (SEQ ID NO: 65) (defined on the right of the plot).

Figure 4. Kinetic analysis of combined S1 and S2 binding site subtilisin mutants versus substrates having variable P1 and P2 residues. The kinetic constants k_{cat}/Km were determined from plots of initial rates versus substrate concentration for the tetrapeptide series succinyl-Ala-Ala-Xaa_2-Xaa_1-pNa (SEQ ID NO: 71), were Xaa_2-Xaa_1 was Lys-Lys (SEQ ID NO: 66), Lys-Arg (SEQ ID NO: 67), Lys-Phe(SEQ ID NO: 62), Pro-Lys (SEQ ID NO: 58), Pro-Phe (SEQ ID NO: 56), or Ala-Phe (SEQ ID NO: 63) (defined on the right of the plot).

Figure 5. Results of hGH-AP fusion protein assay. hGH-AP fusion proteins were constructed, bound to hGHbp-coupled resin, and treated with 0.5 nM N62D/G166D subtilisin in 20 mM Tris-Cl pH 8.2. Aliquots were withdrawn at various times and AP release was monitored by activity assay in comparison to a standard curve. Arrows indicate the cleavage site. The rate of cleavage of fusion proteins containing various substate linkers is shown. Substrates containing a Pro at position P1' are not cleaved.

Figure 6-1 - 6-10. (Collectively referred to herein as Fig. 6). DNA sequence of the phagemid pSS5 containing the N62D/G166D double mutant subtilisin BPN' gene (SEQ ID NO: 1), and translated amino acid sequence for the mutant preprosubtilisin (SEQ ID NO: 2). The pre region is comprised of residues -107 to -78, the pro of residues -77 to -1, and the mature enzyme of residues +1 to +275 (SEQ ID NO: 72). Also shown are restriction sites recognized by endonucleases that require 6 or more specific bases in succession.

Figure 7. Structure of a succinyl-Ala-Ala-Pro-BoroPhe (SEQ ID NO: 69) inhibitor bound to the active site of subtilisin BPN' showing the S1, S2, and S4 binding pocket residues subjected to mutagenesis.

Figure 8. DNA sequence of the N62D/Y104D/G166D triple mutant (SEQ ID NO:74) as well as the translated amino acid sequence (SEQ ID NO:75). The preregion is comprised of residues -107 to -78, the pro residues -77 to -1 and the mature enzyme +1 to +275. The proregion reflects the changes, A(-4)R/A(-2)K,Y(-1)R made in the wild-type processing site to affect expression.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Terms used in the claims and specification are defined as set forth below unless otherwise specified.

The term amino acid or amino acid residue, as used herein, refers to naturally occurring L amino acids or residues, unless otherwise specifically indicated. The commonly used one- and three-letter abbreviations for amino acids are use herein (Lehninger, A. L., *Biochemistry*, 2d ed., pp. 71-92, Worth Publishers, N. Y. (1975)). Basic amino acids are Arg and Lys. Acidic amino acids are Asp and Glu.

Substrates are described in triplet or single letter code as Pn...P2-P1-P1'-P2'...Pn'. The "P1" residue refers to the position proceeding (i.e., N-terminal to) the scissile peptide bond (*i.e.* between the P1 and P1' residues) of the substrate as defined by Schechter and Berger (Schechter, I. and Berger, A., Biochem. Biophys. Res. Commun. 27: 157-162 (1967)). Similarly, the term P1' is used to refer to the position following (i.e., C-terminal to) the scissile peptide bond of the substrate. Increasing numbers refer to the next consecutive position preceding (e.g., P2 and P3) and following (e.g., P2' and P3') the scissile bond. According to the present invention the scissile peptide bond is that bond that is cleaved by the subtilisin variants of the instant invention.

"Subtilisins," "precursor subtilisin" and the like are bacterial carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally occurring subtilisin or a recombinant subtilisin. A series of naturally occurring subtilisins are known to be produced and often secreted by various bacterial species (Siezen, R.J., et al., (1991) Protein Engineering 4:719-737). Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however is histidine-aspartate-serine. Thus, subtilisins as used herein refer to a serine protease having the catalytic triad of subtilisin related proteases.

Generally, subtilisins are serine endoproteases' having molecular weights of about 27,500 which are secreted in large amounts from a wide variety of Bacillus species. The protein sequence of subtilisins have been determined from at least four different species of Bacillus (Markland, F.S., *et al.* (1971) in *The Enzymes*. ed. Boyer P.D., Acad Press, New York, Vol. III, pp. 561-608; and Nedkov, P. *et al.* (1983) Hoppe-Seyler's Z. Physiol. Chem. 364:1537-1540). The three-dimensional crystallographic structure of four subtilisins have been reported (BPN' from *Bacillus amyloliquefaciens*, Hirono et al. (1984) J. Mol. Biol. 178:389-413; subtilisn Carlesberg from *Bacillus licheniformis*, Bode et al., (1986) EMBO J., 5:813-818; thermitase from *Thermoactinomyces vulgaris*, Gros et al., (1989) J. Mol. Biol. 210:347-367; and proteinase K from *Tritirachium album*, Betzel, et al., (1988) Acta Crystallogr., B, 44:163-172). The three dimensional structure of subtilisin BPN' (from *B. amyloliquefaciens*) to 2.5Å resolution has also been reported by Wright, C.S. *et al.* (1969) *Nature* 221:235-242 and Drenth, J. *et al.* (1972) *Eur. J. Biochem.* 26:177-181. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar fold and active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al.

(1972) Biochemistry 11:2439-2449), product complexes (Robertus, J.D., et al. (1972) Biochemistry 11:4293-4303), and transition state analogs (Matthews, D.A., *et al.* (1975) *J. Biol. Chem.* 250:7120-7126 and Poulos, T.L., *et al.* (1976) *J. Biol. Chem.* 251:1097-1103), which have been reported have also provided information regarding the active site and putative substrate binding cleft of subtilisins. In addition, a large number of kinetic and chemical modification studies have been reported for subtilisins (Phillip, M., *et al.* (1983) *Mol. Cell. Biochem.* 51:5-32; Svendsen, I.B. (1976) *Carlsberg Res. Comm.* 41:237-291 and Markland, F.S. *Id.*) as well as at least one report wherein the side chain of methione at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer, D.C., *et al.* (1965) *J. Biol. Chem.* 244 5333-5338).

"Subtilisin variant," "subtilisin mutant" and the like refer to a subtilisin-type serine protease having a sequence which is not found in nature that is derived from a precursor subtilisin according to the present invention. The subtilisin variant has a substrate specificity different from the precursor subtilisin by virtue of amino acid substitutions within the precursor subtilisin amino acid sequence. The term is meant to include subtilisin variants in which the DNA sequence encoding the precursor subtilisin is modified to produce a mutant DNA sequence which encodes the substitution of one or more amino acids in the naturally occurring subtilisin amino acid sequence. Suitable methods to produce such modification include those disclosed in U. S. Patent No. 4,760,025 and 5,371,008 and in EPO Publication No. 0130756 and 0251446.

A change in substrate specificity is defined as a difference between the K_{cat}/Km ratio of the precursor subtilisin and the subtilisin variant. The K_{cat}/Km ratio is a measure of catalytic efficiency. Subtilisin variants with increased or decreased K_{cat}/Km ratios compared to the precursor subtilisin from which they were derived are described herein. Generally, the objective is to secure a variant having a greater, i.e. numerically larger, K_{cat}/Km ratio for a given substrate. A greater K_{cat}/Km ratio for a particular substrate indicates that the variant may be used to more efficiently cleave the target substrate.

The specificity or discrimination between two or more competing substrates is determined by the ratios of k_{cat}/Km (Fersht, A.R., (1985) in Enzyme Structure and Mechanism, W.F. Freeman and Co., N.Y. p. 112). An increase in K_{cat}/Km ratio for one substrate may be accompanied by a reduction in K_{cat}/Km ratio for another substrate. This shift in substrate specificity indicates that the variant subtilisin with the increased K_{cat}/Km ratio for the substrate has utility in cleaving the particular substrate over the precursor subtilisin in, for example, preventing undesirable hydrolysis of a particular substrate in a mixture of substrates.

In general, for a subtilisin variant to have a useful catalytic efficiency for cleavage of a particular substrate the K_{cat}/Km ratio will generally be between 1×10^3 $M^{-1}s^{-1}$ to about 1×10^7 $M^{-1}s^{-1}$. More often, the K_{cat}/Km ratio will be between about 1×10^4 $M^{-1}s^{-1}$ and 1×10^6 $M^{-1}s^{-1}$.

When referring to mutants or variants, the wild type amino acid residue is followed by the residue number and the new or substituted amino acid residue. For example, substitution of D for wild type N in residue position 62 is denominated N62D.

"Subtilisin variants or mutants" are designated in the same manner by using the single letter amino acid code for the wild-type residue followed by its position and the single letter amino acid code of the replacement residue. Multiple mutants are indicated by component single mutants separated by slashes. Thus the subtilisin BPN' variant N62D/G166D is a di-substituted variant in which Asp replaces Asn and Gly at residue positions 62 and 166, respectively, in wild-type subtilisin BPN'.

An amino acid residue of a precursor carbonyl hydrolase is "equivalent" to a residue of *B. amyloliquefaciens* subtilisin if it is either homologous (*i.e.*, corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *B. amyloliquefaciens* subtilisin (*i.e.*, having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly compared to the *B. amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in all subtilisins for which the sequences are known (see *e.g.* Figure 5-C in EPO 0251446). After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (*i.e.*, avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *B. amyloliquefaciens* subtilisin are defined. Alignment of conserved residues should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221, is required.

Equivalent residues homologous at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography, are defined as those for which the atomic coordinates of 2 or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and *B. amyloliquefaciens* subtilisin (N on N, CA on CA, C on C, and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the *B. amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R \text{ factor} = \frac{\sum_h |Fo(h)| - |Fc(h)|}{\sum_h |Fo(h)|}$$

Equivalent amino acid residues of subtilisin BPN', subtilisin Carslberg, thermitase and proteinase K from tertiary structure analysis is provided in, for example, Siezen, et al., (1991) Prot. Eng. 4:719-737.

Equivalent residues which are functionally analogous to a specific residue of *B. amyloliquefaciens* subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *B. amyloliquefaciens* subtilisin as described herein. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography), which occupy an analogous position to the extent that although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the

corresponding side chain atoms of *B. amyloliquefaciens* subtilisin. The three dimensional structures would be aligned as outlined above.

Some of the residues identified for substitution are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a mutant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally occurring sequence. The subtilisin mutants of the present invention include the mature forms of subtilisin mutants as well as the pro- and prepro-forms of such subtilisin mutants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the subtilisin mutants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a subtilisin which when removed results in the appearance of the "mature" form of the subtilisin. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. The preferred prosequence for producing subtilisin mutants, specifically subtilisin BPN' mutants, is the putative prosequence of *B. amyloliquefaciens* subtilisin although other subtilisin prosequences may be used. For example, when the substrate specificity of the precursor subtilisin is altered according to the present invention, this alteration may affect the ability of the variant subtilisin to undergo autolytic cleavage of the naturally occurring prosequence. In order to affect the expression and proper folding of a mature variant subtilisin whose substrate specificity has been altered, it may be necessary to alter the prosequence to correspond to the new or variant substrate specificity.

As an example, the substrate specificity of a particular subtilisin variant N62D/Y104D/G166D is distinct from the precursor subtilisin from which it was derived. The subtilisin variant prefers substrates containing basic residues at substrate positions corresponding to P4, P2, and P1. According to this aspect of the present invention, the precursor prosequence which was efficiently autolysed by the precursor subtilisin is altered to correspond to the substrate specificity of the variant subtilisin. Therefore, for the subtilisin variant N62D/Y104/G166D the prosequence would be altered to contain basic residues at positions -4, -2, and -1.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a subtilisin or to the N-terminal portion of a prosubtilisin which may participate in the secretion of the mature or pro forms of the subtilisin. This definition of signal sequence is a functional one, meant to include all those amino acid sequences, encoded by the N-terminal portion of the subtilisin gene or other secretable carbonyl hydrolases, which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The present invention utilizes such sequences to effect the secretion of the subtilisin mutants as defined herein.

A "prepro" form of a subtilisin mutant consists of the mature form of the subtilisin having a prosequence operably linked to the amino-terminus of the subtilisin and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control termination of

transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in EPO Publication No. 0130756 or 0251446 or U.S. Patent No. 5,371,008 to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the Bacillus strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in EPO Publication No. 0130756 and further described by Yang, M.Y., *et al.* (1984) J. Bacteriol. 160:15-21. Such host cells are distinguishable from those disclosed in PCT Publication No. 03949 wherein enzymatically inactive mutants of intracellular proteases in *E. coli* are disclosed. Other host cells for expressing subtilisin include *Bacillus subtilis* var. I168 (EPO Publication No. 0130756).

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the subtilisin mutants or expressing the desired subtilisin mutant. In the case of vectors which encode the pre or prepro form of the subtilisin mutant, such mutants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor subtilisin may be obtained in accord with the general methods described in U.S. Patent No. 4,760,025 or EPO Publication No. 0130756. As can be seen from the examples disclosed therein, the methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms expressing the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

The cloned subtilisin is then used to transform a host cell in order to express the subtilisin. The subtilisin gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promotor if it is recognized, *i.e.*, transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the subtilisin gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in

antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the subtilisin gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

Once the subtilisin gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor subtilisin. Such modifications include the production of recombinant subtilisin as disclosed in U.S. Patent No. 5,371,008 or EPO Publication No. 0130756 and the production of subtilisin mutants described herein.

Mutant design and preparation.

A. Subtilisin Variants Capable of Cleaving Substrates Having Dibasic Residues.

For the preparation of subtilisin variants capable of cleaving substrates containing dibasic residues, the following analysis was undertaken.

A number of structures have been solved of subtilisin with a variety of inhibitors and transition state analogs bound (Wright, C. S., Alden, R. A. and Kraut, J. (1969) *Nature*, 221:235-242; McPhalen, C.A. and James, N.G. (1988) *Biochemistry*, 27:6582-6598; Bode, W., Papamokos, E., Musil, D., Seemueller, U. and Fritz, M. (1986) *EMBO J.*, 5:813-818; and Bott, R., Ultsch, M., Kossiakoff, A., Graycar, T., Katz, B. and Power, S. (1988) *J. Biol. Chem.*, 263:7895-7906). One of these structures, Figure 1, was used to locate residues that are in close proximity to side chains at the P1 and P2 positions from the substrate. Previous work had shown that replacement residues at positions 156 and 166 in the S1 binding site with various charged residues lead to improved specificity for complementary charged substrates (Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P. and Estell, D. A. (1987) Proc. Natl. Acad. Sci. USA, 84:1219-1223). Although longer range electrostatic effects of substrate specificity have been noted (Russell, A. J. and Fersht, A. R. (1987) *Nature*, 328:496-500) these were generally much smaller than local ones. Therefore, it seemed reasonable that local differences in charge between subtilisin BPN' and the eukaryotic enzymes may account for the differences in specificity.

A detailed sequence alignment of 35 different subtilisin-like enzymes (Siezen, R. J., de Vos, W. M., Leunissen, A. M., and Dijkstra, B. W. (1991) *Prot. Eng.*, 4:719-737) allowed us to identify differences between subtilisin BPN' and the eukaryotic processing enzymes, KEX2, furin and PC2. Within the S1 binding pocket there are a number of charged residues that appear in the pro-hormone processing enzymes and not in subtilisin BPN' (Table 1A).

TABLE 1A

S1 subsite

	125-131[a]	151-157	163-168
Subtilisin BPN'	S L G G P S G (SEQ ID NO: 3)	A A A G N E G (SEQ ID NO: 4)	S T - V G Y P (SEQ ID NO: 5)
Kex2	S W G P A D D (SEQ ID NO: 6)	F A S G N G G (SEQ ID NO: 7)	C N Y D G Y T (SEQ ID NO: 8)
Furin	S W G P E D D (SEQ ID NO: 9)	W A S G N G G (SEQ ID NO: 10)	C N C D G Y T (SEQ ID NO: 11)
PC2	S W G P A D D (SEQ ID NO: 6)	W A S G D G G (SEQ ID NO: 12)	C N C D G Y A (SEQ ID NO: 13)

[a] numbering according to subtilisin BPN' sequence

For example, the eukaryotic enzymes have two conserved Asp residues at 130 and 131 as well as an Asp at 165 that is preceded by insertion of a Tyr or Cys. However, in the region from 151-157, subtilisin BPN' contains a Glu and the eukaryotes a conserved Gly.

In the S2 binding site there were two notable differences in sequence (Table 1B).

TABLE 1B

S2 subsite

	30-35	60-64
Subtilisin BPN'	V I D S G I (SEQ ID NO: 14)	D N N S H (SEQ ID NO: 15)
KEX2	I V D D G L (SEQ ID NO: 16)	S D D Y H (SEQ ID NO: 17)
Furin	I L D D G I (SEQ ID NO: 18)	N D N R H (SEQ ID NO: 19)
PC2	I M D D G I (SEQ ID NO: 20)	W F N S H (SEQ ID NO: 21)

Subtilisin BPB' contains a Ser at position 33 whereas the pro-hormone processing enzymes contain Asp. There is not as clear a consensus in the region of 60-64, but one notable difference is at position 62. This side chain which points directly at the P2 side chain (Figure 1) is Asn in subtilisin BPN', furin and PC2 but Asp in KEX2. Thus, not all substitutions were clearly predictive of the specificity differences.

A variety of mutants were produced to probe and engineer the specificity of subtilisin BPN' using oligonucleotides described in Table 2.

TABLE 2

Oligonucleotides used for site-directed mutagenesis on subtilisin.

Mutant	Oligonucleotide	Specificity Pocket	Activity Expressed
S33D	5'- GCGGTTATCGACG°A°CGGTATCGATTCT -3' (SEQ ID NO: 22)	S2	+
S33K	5'- GCGGTTATCGACAA°A°G°GTATCGATTCT -3' (SEQ ID NO: 23)	S2	+
S33E	5'- GCGGTTATCGACG°A°A°GGTATCGATTCT -3' (SEQ ID NO: 24)	S2	+
N62D	5'- CCAAGACAACG°ACTCTCACGGAA -3' (SEQ ID NO: 25)	S2	+
N62S	5'- CCAAGACAACAG°CTCTCACGGAA -3' (SEQ ID NO: 26)	S2	+
N62K	5'- CCAAGACAACAAA°TCTCACGGAA -3' (SEQ ID NO: 27)	S2	+
G166D	5'-CACTTCCGGCAGCTCG°T°C°G°ACAGTGGA°C°T ACCCTGGC.AAATA-3' (SEQ ID NO: 28) (Inserts Sal I site)	S1	+
G166E	5'-CACTTCCGGCAGCTCG°T°C°G°ACAGTGGA°GT ACCCTGGCAAATA-3' (SEQ ID NO: 29) (Inserts Sal I site)	S1	+
G128P/P129A	5'- TTAACATGAGCCTCGGCC°C°AG°CTA°G°C°GGT TCTGCTGCTTTA -3' (SEQ ID NO: 30) (Inserts Nhe I site)	S1	-
G128P/P129A/ S130D/G131D	5'- TTAACATGAGCCTCGGCC°C°C°G°CGG°A°TGA° TTCTGCTGCTTTAAA -3' (SEQ ID NO: 31) (Inserts Sac II site)	S1	-
T164N/V165D	5'-CGGCAGCTCAAGCA°A°C°G°A°T°GGCTAT°CCT GGCAAATACCCTTCTGTCA -3' (SEQ ID NO: 32) (Inserts BsaBI site)	S1	-

T164Y/V165D	5'-CGGCAGCTCAAGCA*A*C*G*A*T*GGCTAT*CCT GGCAAATACCCTTCTGTCA -3' (SEQ ID NO: 33) (Inserts BsaBI site)	S1	-
T164N-Y(insert)-V165D	5'-ACTTCCGGCAGCTCT*T*C*G*AA*C*T*A*C*G*A* C*GGGTACCCTGGCAAATA-3' (SEQ ID NO: 34) (Inserts BstBI site)	S1	-
N62D/G166D	See individual mutations	S1/S2	-
N62D/G166E	See individual mutations	S1/S2	-

* Asterisks indicate base changes from the pSS5 (wild-type) template.

After producing the mutant plasmids they were transformed into a protease deficient strain of *B. subtilis* (BG2036) that lacks an endogenous gene for secretion of subtilisin. These were then tested for protease activity on skim milk plates.

The first set of mutants tested were ones where segments of the S1 binding site were replaced with sequences from KEX2. None of these segment replacements produced detectable activity on skim milk plates even though variants of subtilisin whose catalytic efficiencies are reduced by as much as 1000-fold do produce detectable halos (Wells, J.A., Cunningham, B.C., Graycar, T.P. and Estell, D.A. (1986) *Philos. Trans. R. Soc. Lond. A*. 317:415-423). We went on to produce single residue substitutions that should have less impact on the stability. These mutants at positions 166 in the S1 site, and 33 and 62 in the S2 site, were chosen based on the modeling and sequence considerations described above. Fortunately all single mutants as well as combination mutants produced activity on skim milk plates and could be purified to homogeneity.

Kinetic analysis of variant subtilisins.

To probe the effects of the G166E and G166D on specificity at the P1 position we used substrates having the form suc-AAPX-pna (SEQ ID NO: 69) where X was either Lys (SEQ ID NO. 58), Arg (SEQ ID NO. 59), Phe (SEQ ID NO. 56), Met (SEQ ID NO. 60) or Gln (SEQ ID NO. 61). The k_{cat}/Km values were determined from initial rate measurements and results reported in Figure 2. Whereas the wild-type enzyme preferred Phe>Met>Lys>Arg>Gln, the G166E preferred Lys~Phe>Arg~Met>Gln, and G166D preferred Lys>Phe~Arg~Met>Gln. Thus, both the acidic substitutions at position 166 caused a shift in preference for basic residues at the P1 site, as previously reported (Wells, J. A., Powers. D. B., Bott, R. R., Graycar, T. P.and Estell, D. A. (1987a), *Proc. Natl. Acad. Sci. USA* 84:1219-1223).

The effects of single and double substitutions in the S2 binding site were analyzed with substrates having the form, suc-Ala-Ala-Xaa-Phe-pna (SEQ ID N0. 70) and are shown in Figure 3. At the P2 position the wild-type enzyme preferred Ala>Pro>Lys>Arg>Asp. In contrast, the S33D preferred Ala>Lys~Arg~Pro>Asp and the N62D preferred Lys>Ala>Arg>Pro>Asp. Although the effects were more dramatic for the N62D mutant, the S33D variant also showed significant improvement toward basic P2 residues and corresponding reduction

in hydrolysis of the Ala and Asp P2 substrates. We then analyzed the double mutant, but found it exhibited the catalytic efficiency of the worse of the two single mutants for each of the substrates tested.

Despite the less than additive effects seen for the two charged substitutions in the S2 site, we decided to combine the best S2 site variant (N62D) with either of the acidic substitutions in the S1 site. The two double mutants, N62D/G166E and N62D/G166D, were analyzed with substrates having the form, suc-AAXX-pna (SEQ ID NO. 71) where XX was either KK (SEQ ID NO. 66), KR (SEQ ID NO. 67), KF (SEQ ID NO. 62), PK (SEQ ID NO. 58), PF (SEQ ID NO. 56) or AF (SEQ ID NO. 63) (Figure 4). The wild-type preference was AF>PF~KF>KK~PK>KR, whereas the double mutants had the preference KK>KR>KF>PK~AF>PF. Thus for the double mutants there was a dramatic improvement toward cleavage of dibasic substrates and away from cleaving the hydrophobic substrates.

The greater than additive effect (or synergy) of these mutants can be seen from ratios of the catalytic efficiencies for the single and multiple mutants. For example, the G166E variant cannot distinguish Lys from Phe at the P1 position. Yet the N62D/G166E variant cleaves the Lys-Lys substrate about 8 times faster than the Lys-Phe substrate. Similarly the G166D cleaves the Lys P1 substrate about 3 times faster than the Phe P1 substrate, but the N62D/G166D double mutant cleaves a Lys-Lys substrate 18 times faster than a Lys-Phe substrate. Thus, as opposed to the reduction in specificity seen for the double mutant in the S2 site, the S1-S2 double mutants enhance specificity for basic residues. It is possible that these two sites bind the dibasic substrates in a cooperative manner analogous to a chelate effect.

Therefore, according to the present invention, subtilisin mutants having a preference for dibasic residues are preferred. According to this aspect of the present invention substitution of amino acids corresponding to amino acids N62 and G166 of subtilisin BPN' produced from *Bacillus amyloliquefaciens* are prepared. In particular, amino acids 62 and 166, or their equivalents, in the precursor subtilisin are substituted with amino acid residues Asp or Glu. Preferred subtilisin variants according to this aspect of the invention include N62D/G166D, N62E/G166E, N62E/G166D, and N62D/G166E variants of subtilisin BPN' and their equivalents.

B. Subtilisin Variants Capable of Cleaving Substrates Having Tribasic Residues

For the preparation of subtilisin variants specific for substrates containing a third basic residue at substrate position P4 we used the crystal structure of subtilisin BPN' complexed with Ala-Ala-Pro-Phe-Boronate(SEQ ID NO: 56) (Figure 7) in combination with sequence alignments of subtilisin BPN', KEX2, Furin, PC2, and P (Table 3) in designing basic specificity into the S1 and S2 and S4 subsites. The two subtilisin BPN' residues that most prominently display their side chains into the S4 pocket are Y104 and I107 (Figure 7).

Sequence alignments of subtilisin BPN' and the mammalian prohormone-processing proteases (Siezen, R. J., de Vos, W. M., Leunissen, A. M., and Dijkstra, B. W. (1991) *Prot. Eng.* 4:719-737) (Table 3) reveal that position 104 is conserved as Asp, and 107 as Glu in the prohormone converting (Arg-P4 specific) enzymes. Therefore these two mutations were introduced either individually or in combination into the dibasic-specific N62D/G166D subtilisin BPN' background (Table 4).

Table 3 Sequence alignments for the S4 site of subtilisins

	S4 Site 100-110	
Subtilisin	GSGQYSWIING	(SEQ ID NO: 77)
KEX2	GDITTEDEAAS	(SEQ ID NO: 78)
Furin	GEVTDAVEARS	(SEQ ID NO: 79)
PC2	PFMTDIIEASS	(SEQ ID NO: 80)
P	GIVTDAIEASS	(SEQ ID NO: 81)

Table 4 describes oligonucleotides used for site-directed mutagenesis, protein regions affected by the mutations, and relative expression of protein for N62D/G166D subtilisin BPN' variants. Bold type indicates base changes from the pSS5 (N62D/G166D) template. For "Protein Expressed," "+" indicates a high level of expression of mature enzyme in crude culture medium, and "-" indicates no enzyme detectable.

TABLE 4

No.	Mutation	Oligonucleotide	Protein Region	Protein Expressed
	Y104D	5'- GGTTCCGGCCAA.GATAGCTGGATCATT -3' (SEQ ID NO: 82)	S4 pocket	-
	I107E	5'- CCAATACAGCTGGGAAATTAACGGAATCG -3' (SEQ ID NO: 83)	S4 pocket	-
	Y104D/I107E	5'- GGTTCCGGCCAAGATAGCTGGGAAATTAACG GAATCGA -3' (SEQ ID NO: 84)	S4 pocket	-
	A(-4)R/ A(-2)K/ Y(-1)R	5'- AAGAAGATCACGTAAGACATAAGCGCGCGC AGTCCGTGC -3' (SEQ ID NO: 85)	Processing site	-
	Y104D/ A(-4)R/ A(-2)K/ Y(-1)R	See individual mutations	S4 pocket + Processing site	+
	I107E/ A(-4)R/ A(-2)K/ Y(-1)R	See individual mutations	S4 pocket + Processing site	-
	Y104D/I107E/ A(-4)R/ A(-2)K/ Y(-1)R	See individual mutations	S4 pocket + Processing site	-

Initial attempts to express the triple mutants in Bacillus were unsuccessful, as indicated by SDS-PAGE of crude supernatants. We reasoned that the source of the expression problem could lie in the fact that correct folding and maturation of subtilisin requires autolytic cleavage of its propeptide (Power, S.D., Adams, R. M., and Wells, J. A. (1986) *Proc. Natl. Acad. Sci. USA* 83, 3096-3100). The processing site in the wild-type enzyme has a sequence that is optimized for the natural substrate preference. AHAY↓A (↓ denotes the site of cleavage). Although the N62D/G166D subtilisin can still autolyze itself with the wild-type processing site, the additional S4 pocket mutations could reduce the cleavage to the point where expression was lowered to a minute level.

To test whether the mutants were expressed poorly due to an inability to autolytically process itself, mutations in the processing site were simultaneously incorporated to accommodate the changes in substrate specificity. Thus the sequence from positions -4 to -1 was changed from AHAY to RHKR in combination with the S4 site mutations. For N62D/Y104D/G166D, high levels of expression could then be achieved providing an indication that the additional Y104D mutation induced an especially strong preference for P4 Arg over Ala. Variants containing the I107E mutation, however, could not be expressed even with the change in the processing site.

Kinetic analysis of variant subtilisins

The mature N62D/Y104D/G166D variant was purified and analyzed for its ability to hydrolyze several tetrapeptide-pNA substrates. Table 5 displays the results along with data for the N62D/G166D mutant and wild-type subtilisin.

Table 5

Kinetic analysis of WT, N62D/G166D, and N62D/Y104D/G166D subtilisin BPN' mutants versus succinyl-tetrapeptide-pNA substrates. Kinetic constants were determined from plots of initial rates versus substrate concentration. Units are as follows: k_{cat}, s^{-1}; Km, μM; and k_{cat}/Km, $M^{-1}s^{-1}$. Standard errors were less than 15%.

Tetrapeptide Sequence, P4-P3-P2-P1												
	AAPF			AAKR			RAKR			KAKR		
Mutant	k_{cat}	Km	k_{cat}/Km	k_{cat}	Km	k_{cat}/Km	k_{cat}	Km	k_{cat}/Km	k_{cat}	Km	k_{cat}/Km
WT	29	110	2.6×10^5	2.8	1700	1.7×10^{3a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
N62D/ G166D	3.4	1800	1.9×10^3	15	41	3.7×10^5	N.D.[b]	N.D.[b]	N.D.[b]	N.D.	N.D.	N.D.
N62D/ Y104D/ G166D	-	-	8.0×10^{0c}	0.27	34	8.1×10^3	11	23	4.9×10^5	9.8	29	3.4×10^5

a Artificially low k_{cat}/Km presumably due to competing cleavage between Lys and Arg

b Biphasic reaction progress curves presumably due to multiple cleavages in the substrate

c Unable to saturate the enzyme, apparent k_{cat}/Km calculated from rates at low substrate concentrations assuming $v = (k_{cat}/Km)(E)(S)$

The tribasic substrates succinyl-RAKR-pNA (SEQ ID NO: 86) and succinyl-KAKR-pNA (SEQ ID NO: 87) were hydrolyzed with high catalytic efficiency (k_{cat}/Km) by the triple mutant, at a level similar to wild-type subtilisin versus one of its best substrates, succinyl-AAPF-pNA (SEQ ID NO: 56). In contrast, the dibasic substrate succinyl-AAKR-pNA (SEQ ID NO: 67) was hydrolyzed 60-fold less efficiently, mostly due to dimunition of k_{cat}. This indicates a dramatic specificity change from the wild-type preference at P4, at which hydrophobic residues are strongly favored over charged side chains (Grøn, H. and Breddam, K. (1992) *Biochemistry* 31, 8967-8971). In fact N62D/G166D subtilisin appears to cleave at an alternate site in the succinyl-RAKR-pNA (SEQ ID NO: 86) substrate, indicating that Arg was not accepted in its wild-type S4 site.

The large magnitude of the combined specificity changes in the N62D/Y104D/G166D variant is evidenced by its strong discrimination against substrates that are preferred by the wild-type enzyme. For example, succinyl-AAPF-pNA (SEQ ID NO: 56) is hydrolyzed 6×10^6-fold less efficiently than succinyl-RAKR-pNA (SEQ ID NO: 86). Clearly, the S4 site mutation greatly improves upon the discriminatory power of the parent dibasic-specific N62D/G166D subtilisin, where the ratio of catalytic efficiency for succinyl-AAKR-pNA versus succinyl-AAPF-pNA is 1.9×10^2. The improvement in discrimination (310-fold) is also higher than would be predicted from the data for hydrolysis of succinyl-RAKR-pNA (SEQ ID NO: 86) versus succinyl-AAKR-pNA (SEQ ID NO: 67) by the triple mutant (a 60-fold effect).

Therefore in order to produce subtilisin variants capable of cleaving substrates containing basic residues at positions P4, P2, and P1, additional site specific substitutions are made in the dibasic specific subtilisin variants. According to this aspect of the invention, substitution of the amino acid corresponding to Y104 of subtilisin BPN' produced by *Bacillus Amyloliquefaciens*, i.e., amino acid 104 of subtilisin BPN' or its equivalent, produces a variant having substantially altered substrate specificity. In a preferred embodiment of the present invention amino acids corresponding to N62, Y104, and G166 of subtilisin BPN' are substituted with acidic amino acids, preferably Asp and Glu and most preferably Asp. Subtilisin BPN' variants N62D/Y104D/G166D, N62D/Y104E/G166D, N62E/Y104D/G166E, N62E/Y104E/G166E, N62E/Y104D/G166D, N62E/Y104E/G166D, N62D/Y104E/G166E, and N62D/Y104D/G166E, and there equivalents are preferred. Most preferred among this group of subtilisin variants are the N62D/Y104D/G166D subtilisin BPN' variants and their equivalents.

Mutagenesis and Synthetic Techniques

Various techniques are available which may be employed to produce mutant DNA, which can encode the subtilisin variants of the present invention. For instance, it is possible to derive mutant DNA based on naturally occurring DNA sequences that encode for changes in an amino acid sequence of the resultant protein relative to a precursor subtilisin. These mutant DNA can be used to obtain the variants of the present invention.

According to the invention, specific residues of *B. amyloliquefaciens* subtilisin are identified for substitution. These amino acid residue position numbers refer to those assigned to the *B. amyloliquefaciens* subtilisin sequence (see the mature sequence in Fig. 1. of U.S. Patent No. 4,760,025). The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor subtilisins

containing amino acid residues which are equivalent, as defined herein, to the particular identified residues in *B. amyloliquefaciens* subtilisin. Equivalent amino acids can be found in, for instance, subtilisn Carlesberg from *Bacillus licheniformis*, Bode et al., (1986) EMBO J., 5:813-818; thermitase from *Thermoactinomyces vulgaris*, Gros et al., (1989)J. Mol. Biol. 210:347-367; and proteinase K from *Tritirachium album*. Betzel, et al., (1988) Acto Crysollogr., B, 44:163-172) as described by Siezen et al., (1991) Prof. Eng., 4: 719-737).

By way of illustration, with expression vectors encoding the precursor subtilisin in hand (see for example U.S. Patent No 4,760,025) site specific mutagenesis (Kunkel et al., (1991) Methods Enzymol. 204:125-139; Carter, P., et al., (1986) Nucl. Acids. Res. 13:4331; Zoller, M. J. et al., (1982) Nucl. Acids Res. 10:6487), cassette mutagenesis (Wells, J. A., et al., (1985) Gene 34:315), restriction selection mutagenesis (Wells, J. A., et al., (1986) Philos. Trans, R. Soc. London Ser A 317, 415) or other known techniques may be performed on the DNA. The mutant DNA can then be used in place of the parent DNA by insertion into the appropriate expression vectors. Growth of host bacteria containing the expression vectors with the mutant DNA allows the production of variants which can be isolated as described herein.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing the variants of the present invention. This technique is well known in the art as described by Adelman et al., (1983) DNA, 2:183. Briefly, the native or unaltered DNA of a precursor subtilisin, for instance subtilisin BPN', is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the precursor.

After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as those described by Crea et al. (1987) Proc. Natl. Acad. Sci. USA, 75:5765. Exemplary oligonucleotide sequences for introducing amino acid changes into precursor subtilisin BPN' are provided in Tables 2 and 4.

Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the variant form of the subtilisin, and the other strand (the original template) encodes the native, unaltered sequence of the precursor subtilisin. This heteroduplex molecule is then transformed into a suitable host cell. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to

identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(αS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(αS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell as described above.

DNA encoding variants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

Cleavage of a Fusion Proteins With Subtilisin Variants

A fusion protein is any polypeptide that contains within it an affinity domain (AD) that usually aids in protein purification, a protease cleavage sequence or substrate linker (SL), which is cleaved by a protease and a protein product of interest (PP). Such fusion proteins are generally expressed by recombinant DNA technology. The genes for fusion proteins are designed so that the SL is between the AD and PP. These usually take the form AD-SL-PP such that the domain closest to the N-terminus is AD and PP is closest to the C-terminus.

Examples of AD would include, glutathione-S-transferase which binds to glutathione, protein A (or derivatives or fragments thereof) which binds IgG molecules, poly-histidine sequences, particularly (His)6 (SEQ ID NO: 51) that bind metal affinity columns, maltose binding protein that binds maltose, human growth hormone that binds the human growth hormone receptor or any of a variety of other proteins or protein domains that can bind to an immobilized affinity support with an association constant (Ka) of $>10^5$ M^{-1}.

The SL can be any sequence which is cleaved by the subtilisin variants of the present invention. In preparations where the variant N62D/Y104D/G166D or its equivalent are used the SL can be any sequence, preferably at least 4 amino acids, in which the P4, P2, and P1 residues are basic residues. Therefore a SL linker is employed of the general formula P4-P3-P2-P1 wherein P4, P2, and P1 are basic amino acid residues. Preferred SLs according to this aspect of the invention include Lys-Ala-Lys-Arg (SEQ ID NO: 87) and Arg-Ala-Lys-Arg (SEQ ID NO: 86).

Likewise, where the N62D/G166D subtilisin variant is contemplated the SL preferably contains di-basic residues. For the variants capable of cleaving substrates containing dibasic residues the SL should be at least four residues and preferably contain a large hydrophobic residue at P4 (such as Leu or Met) and dibasic residues at P2 and P1 (such as Arg and Lys). A particularly good substrate is Leu-Met-Arg-Lys- (SEQ ID NO: 52), but a variety of other sequences may work including Ala-Ser-Arg-Arg (SEQ ID NO: 50) and even Leu-Thr-Ala-Arg (SEQ ID NO 53).

It is often useful that the SL contain a flexible segment on its N-terminus to better separate it from the AD and PP. Such sequences include Gly-Pro-Gly-Gly (SEQ ID NO: 54) but can be as simple as Gly-Gly or Pro-Gly. Thus, an example of a particularly good SL would have the sequence Gly-Pro-Gly-Gly-Leu-Met-Arg-Lys (SEQ ID NO: 88) in the case of subtilisin variants capable of cleaving substrates containing dibasic amino acids, or `Gly-Pro-Gly-Gly-Lys-Ala-Lys-Arg` (SEQ ID NO: 89). This sequence would be inserted between the AD and PP domains.

The PP can be virtually any protein or peptide of interest but preferably should not have a Pro, Ile, Thr, Val, Asp or Glu as its first residue (P1'), or Pro or Gly at the second residue (P2') or Pro at the third residue (P3'). Such residues are poor substrates for the enzyme and may impair the ability of the subtilisins variant to cleave the SL sequence.

The conditions for cleaving the fusion protein are best done in aqueous solution, although it should be possible to immobilize the enzyme and cleave the soluble fusion protein. It may also be possible to cleave the fusion protein as it remains immobilized on a solid support (*e.g.* bound to the solid support through AD) with the soluble subtilisin variant. It is preferable to add the enzyme to the fusion protein so that the enzyme

is less than one part in 100 (1:100) by weight. A good buffer is 10-50mM Tris (pH 8.2) in 10mM NaCl. A preferable temperature is about 25°C although the enzyme is active up to 65°C. The extent of cleavage can be assayed by applying samples to SDS-PAGE. Generally suitable conditions for using the subtilisin variants of this invention do not depart substantially from those known in the art for the use of other subtilisins.

EXAMPLES

In the examples below and elsewhere, the following abbreviations are employed: subtilisin BPN', subtilisin from *Bacillus amyloliquefaciens*; Boc-RVRR-MCA (SEQ ID NO. 73), N-t-butoxy carbonyl-arginine-valine-arginine-arginine-7-amido-4-methyl coumarin; suc-Ala-Ala-Pro-Phe-pna (SEQ ID NO. 56), N-succinyl-alanine-alanine-proline-phenylalanyl-p-nitroanalide (SEQ ID NO. 56); hGH, human growth hormone; hGHbp, extracellular domain of the hGH receptor; PBS, phosphate buffered saline; AP, alkaline phosphatase;

Example 1

Construction and Purification of Subtilisin Mutants.

Site-directed mutations were introduced into the subtilisin BPN' gene cloned into the phagemid pSS5 (Wells, J. A., Ferrari, E., Henner, D. J., Estell, D.A. and Chen, E. Y. (1983) *Nucl. Acids Res.* 11:7911-7929). Single-stranded uracil-containing pSS5 template was prepared and mutagenesis performed using the method of Kunkel (Kunkel, T. A., Bebenek, K and McClary, J. (1991) *Methods Enzymol.* 204:125-139). For example, the synthetic oligonucleotide N62D,

(5'- CCAAGACAACG°ACTCTCACGGAA -3') (SEQ ID NO. 25)

in which the asterisk denotes a mismatch to the wild-type sequence, was used to construct the N62D mutant. The oligonucleotide was first phosphorylated at the 5' end using T4 polynucleotide kinase according to a described procedure (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor, N.Y.). The phosphorylated oligonucleotide was annealed to single-stranded uracil-containing pSS5 template, the complementary DNA strand was filled in with deoxynucleotides using T7 polynucleotide kinase, and the resulting nicks ligated using T4 DNA ligase according to a previously described procedure (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor, N.Y.). Heteroduplex DNA was transformed into the E. coli host JM101(Yanish-Perron, C., Viera, J., and Messing, J. (1985) Gene 33: 103-199), and putative mutants were confirmed by preparation and dideoxy nucleotide sequencing of single stranded DNA (Sanger, F., Nicklen, S. and Coulson, A. R. (1977)*Proc. Natl. Acad. Sci. USA* 74:5463-5467) according to the SEQUENASE® protocol (USB Biochemicals). Mutant single-stranded DNA was then retransformed into JM101 cells and double stranded DNA prepared according to a previously

described procedure (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor, N.Y.). For other mutations also requiring the use of one primer, the oligonucleotides used are listed in Table 2. For several of these oligonucleotides, additional silent mutations emplacing new restriction sites were simultaneously introduced to provide an alternative verification of mutagenesis.

To construct the double mutants N62D/G166D, and N62D/G166E, pSS5 DNA containing the N62D mutation was produced in single-stranded uracil-containing form using the Kunkel procedure (Kunkel, T. A., Bebenek, K and McClary, J. (1991) *Methods Enzymol.* 204, 125-139). This mutant DNA was used as template for the further introduction of the G166D or G166E mutations, using the appropriate oligonucleotide primers (see sequences in Table 2), following the procedures described above.

To construct the triple mutants, such as N62D/Y104D/G166D, pSS5 DNA containing the N62D/G166D mutation or other appropriate double mutation, was produced in single-stranded uracil-containing form using the Kunkel procedure (Kunkel, T. A., Bebenek, K and McClary, J. (1991) Methods Enzymol. 204, 125-139). This mutant DNA was used as template for the further introduction of the Y104D mutations, using the appropriate oligonucleotide primers (see sequences in Table 4), following the procedures described above.

For expression of the subtilisin BPN' mutants, double stranded mutant DNA was transformed into a protease-deficient strain (BG2036) of *Bacillus Subtilis* (Yang, M. Y., Ferrari, E. and Henner, D. J. (1984)*Journal of Bacteriology* 160:15-21) according to a previous method (Anagnostopolouus, C. and Spizizen, J. (1961) *Journal of Bacteriology* 81:741-746) in which transformation mixtures were plated out on LB plus skim milk plates containing 12.5 µg/mL chloramphenicol. The clear halos indicative of skim milk digestion surrounding transformed colonies were noted to roughly estimate secreted protease activity.

The transformed BG2036 strains were cultured by inoculating 5 mL of 2xYT media (Miller, J. H., (1972) in "Experiments in Molecular Genetics," Cold Spring Harbor, N.Y.) containing 12.5 µg/mL chloramphenicol and 2 mM $CaCl_2$ at 37 °C for 18-20 h, followed by 1:100 dilution in the same medium and growth in shake flasks at 37 °C for 18-22 h with vigorous aeration. The cells were harvested by centrifugation (6000g, 15 min, 4°C), and to the supernatant 20mM (final) $CaCl_2$ and one volume of ethanol (-20°C) were added. After 30 min at 4°C, the solution was centrifuged (12,000g, 15 min, 4°C), and one volume of ethanol (-20°C) added to the supernatant. After 2 h at -20°C, the solution was centrifuged (12,000g, 15 min, 4°C) and the pellet resuspended in and dialyzed against MC (25 mM 2-(N-Morpholino)ethanesulfonic acid (MES), 5 mM $CaCl_2$ at pH 5.5) overnight at 4°C. The dialysate was passed through a 0.22 µm syringe filter and loaded onto a mono-S cation exchange column run by an FPLC system (Pharmacia Biotechnology). The column was washed with 20 volumes of MC and mutant subtilisin eluted over a linear gradient of zero to 0.15 M NaCl in MC, all at a flow rate of 1 mL/min. Peak fractions were recovered and the subtilisin mutant quantitated by measuring the absorbance at 280 nm (E_{280} 0.1% = 1.17) (Matsubara, H.; Kasper, C B.; Brown, D. M.; and Smith, E. L. (1965)*J. Biol. Chem.*, 240:1125-1130.)

Example 2

Kinetic Characterizations

Subtilisins were assayed by measuring the initial rates of hydrolysis of *p*-nitroanilide tetrapeptide substrates in 0.4 mL 20 mM Tris-Cl pH 8.2, 4 % (v/v) dimethyl sulfoxide at (25 ± 0.2)°C as described previously (Estell, D. A., Graycar, T. P., Miller, J. V., Powers, D. B., Burnier, J. P., Ng, P. G. and Wells, J. A. (1986)*Science* 233:659-663). Enzyme concentrations $(E)_0$ were determined spectrophotometrically using $E_{280\,nm}$ 0.1% = 1.17 (Matsubara, H.; Kasper, C B.; Brown, D. M.; and Smith, E. L. (1965)J. Biol. Chem.,240:1125-1130.), and were typically 5-50 nM in reactions. Initial rates were determined for nine to twelve different substrate concentrations over the range of 0.001-2.0 mM. Plots of initial rates (v) versus substrate concentration (S) were fitted to the Michaelis-Menton equation,

$$v = \frac{k_{cat}(E)_0((S))}{Km + (S)}$$

to determine the kinetic constants k_{cat} and Km (Fersht, A. in "Enzyme Structure and Mechanism", Second edition, Freeman and Co., N.Y.) using the program Kaleidagraph (Synergy Software, Reading, PA).

Example 3

Substrate Phage

Substrate phage selections were performed as described by Matthews and Wells (Matthews, D. J. and Wells, J. A. (1993)*Science* 260:1113-1117), with minor modifications. Phage sorting was carried out using a library in which the linker sequence between the gene III coat protein and a tight-binding variant of hGH was GPGGX$_3$GGPG (SEQ ID NO. 52). The library contained 2 x 10^6 independent transformants. Phage particles were prepared by infecting 1 mL of log phase 27C7 (F'/tet^R/Ompt$^-$degP$^-$) *Escherichia coli* with approximately 10^8 library phage for 1 h at 37°C, followed by 18-24 h of growth in 25 mL 2YT medium containing 10^{10} M13K07 helper phage and 50 µg/mL carbenicillin at 37°C. Wells of a 96-well Nunc Maxisorb microtiter plate were coated with 2 µg/mL of hGHbp in 50 mM $NaHCO_3$ at pH 9.6 overnight at 4°C and blocked with PBS (10 mM sodium phosphate at pH 7.4 nd 150 mM NaCl) containing 2.5% (w/v) skim milk for 1 h at room temperature. Between 10^{11} and 10^{12} phage in 0.1 mL 10 mM tris-Cl (pH 7.6), 1 mM EDTA, and 100 mM NaCl were incubated in the wells at room temperature for 2 h with gentle agitation. The plate was washed first with 20 rinses of PBS plus 0.05% Tween 20 and then twice with 20 mM tris-Cl at pH 8.2. The N62D/G166D subtilisin was added in 0.1 mL of 20 mM tris-Cl at pH 8.2 and protease sensitive phage were eluted after a variable reaction time. The concentration of protease and incubation times for elution of sensitive phage were decreased gradually over the course of sorting procedure to increase selectivity, with protease concentrations of 0.2 nM (rounds 1-3) and 0.1 nM (rounds 4-9), and reaction times of 5 min (rounds 1-6), 2.5 min (round 7), 40 s (round 8) and 20 s (round 9). Control wells in which no protease was added were also included in each round. For the resistant phage pool, the incubation time with protease remained constant at 5 min. The wells were then washed ten times with PBS plus 0.05% Tween 20 and resistant phage eluted by treatment with 0.1 mL of 0.2 M glycine at pH 2.0 in PBS plus 0.05% Tween

20 for 1 min at room temperature. Protease sensitive and resistant phage pools were titered and used to infect log phase 27C7 cells for 1 h at 37°C, followed by centrifugation at 4000 rpm, removal of supernatant, and resuspension in 1 mL 2YT medium. The infected cells were then grown 18-24 h in the presence of helper phage as described above and the process repeated 9 times. Selected substrates were introduced into AP fusion proteins and assayed for relative rates of cleavage as described by Matthews and Wells (Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994)Protein Science 3:1197-1205 and Matthews, D. J. and Wells, J. A. (1993)*Science* 260:1113-1117), except that the cleavage reactions were performed in 20 mM Tris-Cl at pH 8.2.

Example 4

Substrate phage selection and cleavage of a fusion protein

Subtilisin has the capability to bind substrates from the P4 to P3' positions (McPhalen, C. A. and James, N. G. (1988) *Biochemistry* 27:6582-6598 and Bode, W., Papamokos, E., Musil, D., Seemueller, U. and Fritz, M. (1986) *EMBO J.* 5:813-818). Given this extensive binding site and the apparent cooperative nature in the way the substrate can bind the enzyme we wished to explore more broadly the substrate preferences for the enzyme. To do this we utilized the substrate phage selection (Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994)Protein Science 3:1197-1205 and Matthews, D. J. and Wells, J. A. (1993)*Science* 260:1113-1117) described in Example 3. In this method a five-residue substrate linker that was flanked by di-glycine residues is inserted between an affinity domain (in this case a high affinity variant of hGH) and the carboxy-terminal domain of gene III, a minor coat protein displayed on the surface of the filamentous phage, M13. The five residue substrate linker is fully randomized to generate a library of 20^5 different protein sequence variants. These are displayed on the phage particles which are allowed to bind to the hGHbp. The protease of interest was added and if it cleaved the phage particle at the substrate linker it released that particle. The particles released by protease treatment can be propagated and subjected to another round of selection to further enrich for good protease substrates. Sequences that are retained can also be propagated to enrich for poor protease substrates. By sequencing the isolated phage genes at the end of either selection one can identify good and poor substrates for further analysis.

We chose to focus on the subtilisin BPN' variant N62D/G166D as it was slightly better at discriminating the synthetic dibasic substrates from the others. We subjected the substrate phage library to nine rounds of selection with the subtilisin variant and isolated clones that were either increasingly sensitive or resistant to cleavage. Of twenty-one clones sequenced from the sensitive pool eighteen contained dibasic residues, eleven of which had the substrate linker sequence Asn-Leu-Met-Arg-Lys (SEQ ID NO: 35) (Table 6).

TABLE 6

Substrate phage sequences sensitive or resistant to N62D/G166D subtilisin from a GG-xxxxx-GG library after 9 rounds of selection[a].

Protease Sensitive Pool

No Basic Sites (0)	Monobasic Sites (3)	Dibasic Sites (18)
	N L T A R(3) (SEQ ID NO: 34)	N L M R X(11) (SEQ ID NO: 35
		T A S R R(4) (SEQ ID NO: 36
		L T R R S (SEQ ID NO: 37
		A L S R X (SEQ ID NO: 38)
		L M L R X (SEQ ID NO: 39)

Protease Resistant Pool

No Basic Sites (7)	Monobasic Sites (2)	Dibasic Sites (1)
A S T H F (SEQ ID NO: 40)	Q X P N F (SEQ ID NO: 41)	R X P T H (SEQ ID NO: 42)
I Q Q Q Y (SEQ ID NO: 43)	R P G A M (SEQ ID NO: 44)	
Q G E L P (SEQ ID NO: 45)		
A P D P T (SEQ ID NO: 46)		
Q L L E H (SEQ ID NO: 47)		
V N N N H (SEQ ID NO: 48)		
A Q S N L (SEQ ID NO: 49)		

[a] Numbers in parentheses indicate the number of times a particular DNA sequence was isolated.

Three (3) of the sensitive sequences were monobasic, Asn-Leu-Thr-Ala-Arg (SEQ ID NO: 34). It is known that subtilisin has a preference for hydrophobic residues at the P4 position. If these and the other selected substrates were indeed cleaved after the last basic residue they all would have a Leu, Met or Ala at the P4 position. Almost no basic residues were isolated in the protease resistant pool and those that were had a Pro following the mono- or dibasic residue. It is known that subtilisin does not cleave substrates containing

Pro at the P1' position (Carter, P., Nilsson, B., Burnier, J., Burdick, D. and Wells, J. A. (1989) *Proteins: Struct., Funct., Genet.* 6:240-248). Thus, di-basic substrates where highly selected and these had the additional feature of Leu, Met or Ala at the P4 position.

Example 5

Cleavage of Substrate Linkers

We wished to analyze how efficiently the most frequently selected sequences were cleaved in the context of a fusion protein. For this we applied an alkaline phosphatase-fusion protein assay (Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994)*Protein Science* 3:1197-1205 and Matthews, D. J. and Wells, J. A. (1993)*Science* 260:1113-1117). The hGH substrate linker domains were excised from the phage vector by PCR and fused in front of the gene for *E. coli* AP. The fusion protein was expressed and purified on an hGH receptor affinity column. The fusion protein was bound to the hGH receptor on a plate and treated with the subtilisin variant. The rate of cleavage of the fusion protein from the plate was monitored by collecting soluble fractions as a function of time and assaying for AP activity (Figure 5). The most frequently isolated substrate sequence, Asn-Leu-Met-Arg-Lys (SEQ ID NO: 35) was cleaved about ten times faster than the next most frequently isolated clones (Thr-Ala-Ser-Arg-Arg (SEQ ID NO: 36) and Asn-Leu-Thr-Ala-Arg (SEQ ID NO: 34). The cleaved AP products were also recovered and subjected to N-terminal sequencing to determine the sites of cleavage (Figure 5), cleavage site denoted by ⇓). In all three fusion proteins, this site was immediately following the dibasic or monobasic site according to the mutant subtilisin design. We also tested the dibasic sequence isolated from the resistant pool, namely Arg-Lys-Pro-Thr-His (SEQ ID NO: 42). We observed no detectable cleavage above background for this substrate during the assay.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

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All references cited herein are expressly incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: SUBTILISIN VARIANTS CAPABLE OF CLEAVING SUBSTRATES CONTAINING BASIC RESIDUES

(iii) NUMBER OF SEQUENCES: 89

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/398028
(B) FILING DATE: 03-MAR-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kubinec, Jeffrey S.
(B) REGISTRATION NUMBER: 36,575
(C) REFERENCE/DOCKET NUMBER: P0936P1PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-8228
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8119 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCNGGT CTACTAAAAT ATTATTCCAT ACTATACAAT TAATACACAG 50

AATAATCTGT CTATTGGTTA TTCTGCAAAT GAAAAAAAGG AGAGGATAAA 100

GA GTG AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT 138
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe
-107 -105 -100

GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA 177
Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr

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-95                 -90                 -85

TCC TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG 216
Ser Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys
        -80                 -75                 -70

AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG 255
Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
                -65                 -60

AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC 294
Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly
    -55                 -50                 -45

GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA GCT 333
Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala
            -40                 -35

TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA 372
Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
-30                 -25                 -20

AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT CAC GTA 411
Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val
        -15                 -10                 -5

GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA 450
Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser
                1                   5

CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA GGC TAC ACT 489
Gln Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr
 10                  15                  20

GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC 528
Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile
         25                  30                  35

GAT TCT TCT CAT CCT GAT TTA AAG GTA GCA GGC GGA GCC 567
Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
                40                  45

AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC 606
Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn
     50                  55                  60

GAC TCT CAC GGA ACT CAC GTT GCC GGC ACA GTT GCG GCT 645
Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala
            65                  70

CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC 684
Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser
 75                  80                  85

GCA TCA CTT TAC GCT GTA AAA GTT CTC GGT GCT GAC GGT 723
Ala Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly
         90                  95                  100

TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG 762
Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp
                105                 110

GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC CTC 801
Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu
    115                 120                 125
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GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT 840
Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val
            130                 135

GAT AAA GCC GTT GCA TCC GGC GTC GTA GTC GTT GCG GCA 879
Asp Lys Ala Val Ala Ser Gly Val Val Val Val Ala Ala
140                 145                 150

GCC GGT AAC GAA GGC ACT TCC GGC AGC TCG TCG ACA GTG 918
Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val
        155                 160                 165

GAC TAC CCT GGC AAA TAC CCT TCT GTC ATT GCA GTA GGC 957
Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly
                170                 175

GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC 996
Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser
    180                 185                 190

GTA GGA CCT GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT 1035
Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser
            195                 200

ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC 1074
Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr
205                 210                 215

AAC GGT ACC TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG 1113
Asn Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala
    220                 225                 230

GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC 1152
Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn
                235                 240

ACT CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA 1191
Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
    245                 250                 255

CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC 1230
Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
            260                 265

GTA CAG GCG GCA GCT CAG TA AAACATAAAA AACCGGCCTT 1270
Val Gln Ala Ala Ala Gln
270                 275

GGCCCCGCCG GTTTTTTATT ATTTTTCTTC CTCCGCATGT TCAATCCGCT 1320

CCATAATCGA CGGATGGCTC CCTCTGAAAA TTTTAACGAG AAACGGCGGG 1370

TTGACCCGGC TCAGTCCCGT AACGGCCAAG TCCTGAAACG TCTCAATCGC 1420

CGCTTCCCGG TTTCCGGTCA GCTCAATGCC GTAACGGTCG GCGGCGTTTT 1470

CCTGATACCG GGAGACGGCA TTCGTAATCG GATCCGGAAA TTGTAAACGT 1520

TAATATTTTG TTAAAATTCG CGTTAAATTT TTGTTAAATC AGCTCATTTT 1570

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AAAGAACGTG GACTCCAACG TCAAAGGGCG AAAAACCGTC TATCAGGGCT 1720
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ATGGCCCACT ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG 1770

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TTGACGGGGA AAGCCGGCGA ACGTGGCGAG AAAGGAAGGG AAGAAAGCGA 1870

AAGGAGCGGG CGCTAGGGCG CTGGCAAGTG TAGCGGTCAC GCTGCGCGTA 1920

ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCCGGATC 1970

NGATCCGACG CGAGGCTGGA TGGCCTTCCC CATTATGATT CTTCTCGCTT 2020

CCGGCGGCAT CGGGATGCCC GCGTTGCAGG CCATGCTGTC CAGGCAGGTA 2070

GATGACGACC ATCAGGGACA GCTTCAAGGA TCGCTCGCGG CTCTTACCAG 2120

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GACCTGAATG GAAGCCGGCG GCACCTCGCT AACGGATTCA CCACTCCAAG 2320

AATTGGAGCC AATCAATTCT TGCGGAGAAC TGTGAATGCG CAAACCAACC 2370

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CGGGAAGCGT GGCGCTTTCT CAATGCTCAC GCTGTAGGTA TCTCAGTTCG 2670

GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT GTGCACGAAC CCCCCGTTCA 2720

GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG 2770

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AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA 2870

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CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC 2970

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AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG 3070

CTCAGTGGAA CGAAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA 3120

AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC 3170

AATCTAAAGT ATATATGAGT AAACTTGGTC TGACAGTTAC CAATGCTTAA 3220

TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT 3270

GCCTGACTCC CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC 3320

TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG 3370

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CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC 3470

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GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG 4170

CGCACATTTC CCCGAAAAGT GCCACCTGAC GTCTAAGAAA CCATTATTAT 4220

CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC TTTCGTCTTC 4270

AAGAATTAAT TCCTTAAGGA ACGTACAGAC GGCTTAAAAG CCTTTAAAAA 4320

CGTTTTTAAG GGGTTTGTAG ACAAGGTAAA GGATAAAACA GCACAATTCC 4370

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AGTGTATCAA ACTGCTAAAT CGGTAGAAGC CCAAACGTTC CACGATGCGA 6270

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AATATTCGTG TGCAAGGACC GACAACATTT CTACCATCCT TGACTGTACA 6370

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CGACTCTGAG AAACTTCTGG AATCGCTAGA GAATTTCTGG AATGGGATTC 6570

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CCATTTTGAA CGATGACCTC TAATAATTGT TAATCATGTT GGTTACGTAT 6670

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GCTCAAATCC TTTAAAAAAA CACAAAAGAC CACATTTTTT AATGTGGTCT 6970

TTATTCTTCA ACTAAAGCAC CCATTAGTTC AACAAACGAA AATTGGATAA 7020

AGTGGGATAT TTTTAAAATA TATATTTATG TTACAGTAAT ATTGACTTTT 7070

AAAAAAGGAT TGATTCTAAT GAAGAAAGCA GACAAGTAAG CCTCCTAAAT 7120

TCACTTTAGA TAAAAATTTA GGAGGCATAT CAAATGAACT TTAATAAAAT 7170

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AACAAACGAC TTTTAGTATA ACCACAGAAA TTGATATTAG TGTTTTATAC 7270

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ATGGTTCGGG GAAATTGTTT CCCAAAACAC CTATACCTGA AAATGCTTTT 7570

TCTCTTTCTA TTATTCCATG GACTTCATTT ACTGGGTTTA ACTTAAATAT 7620

CAATAATAAT AGTAATTACC TTCTACCCAT TATTACAGCA GGAAAATTCA 7670

TTAATAAAGG TAATTCAATA TATTTACCGC TATCTTTACA GGTACATCAT 7720

TCTGTTTGTG ATGGTTATCA TGCAGGATTG TTTATGAACT CTATTCAGGA 7770

ATTGTCAGAT AGGCCTAATG ACTGGCTTTT ATAATATGAG ATAATGCCGA 7820

CTGTACTTTT TACAGTCGGT TTTCTAATGT CACTAACCTG CCCCGTTAGT 7870

TGAAGAAGGT TTTTATATTA CAGCTCCAGA TCCATATCCT TCTTTTTCTG 7920

AACCGACTTC TCCTTTTTCG CTTCTTTATT CCAATTGCTT TATTGACGTT 7970

GAGCCTCGGA ACCCNTATAG TGTGTTATAC TTTACTTGGA AGTGGTTGCC 8020

GGAAAGAGCG AAAATGCCTC ACATTTGTGC CACCTAAAAA GGAGCGATTT 8070

ACATATGAGT TATGCAGTTT GTAGAATGCA AAAAGTGAAA TCAGGATCN 8119

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 382 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala
-107  -105              -100                  -95

Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser Ser Ala Gln Ala
      -90               -85                   -80

Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys
      -75               -70                   -65

Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys Lys Asp Val Ile
      -60               -55                   -50

Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp
      -45               -40                   -35

Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
      -30               -25                   -20

Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Ala His
      -15               -10                    -5

Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala
        1                 5                     10

Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val
       15                20                    25

Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys
       30                35                    40

Val Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe
       45                50                    55

Gln Asp Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala
       60                65                    70

Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala
       75                80                    85

Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln
       90                95                   100

Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn
      105               110                   115

Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala
      120               125                   130

Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val
      135               140                   145

Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser
      150               155                   160

Thr Val Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly
      165               170                   175

Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly
      180               185                   190

Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
      195               200                   205
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Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala
210 215 220

Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His
225 230 235

Pro Asn Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr
240 245 250

Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile
255 260 265

Asn Val Gln Ala Ala Ala Gln
270 275

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Leu Gly Gly Pro Ser Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Ala Ala Gly Asn Glu Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Thr Val Gly Tyr Pro
1 5 6

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Trp Gly Pro Ala Asp Asp
1 5 7

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Phe Ala Ser Gly Asn Gly Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Cys Asn Tyr Asp Gly Tyr Thr
1 5 7

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Trp Gly Pro Glu Asp Asp
1 5 7

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Trp Ala Ser Gly Asn Gly Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Cys Asn Cys Asp Gly Tyr Thr
1 5 7

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Trp Ala Ser Gly Asp Gly Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Cys Asn Cys Asp Gly Tyr Ala
1 5 7

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Val Ile Asp Ser Gly Ile
1 5 6

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asp Asn Asn Ser His
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ile Val Asp Asp Gly Leu
1 5 6

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Asp Asp Tyr His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ile Leu Asp Asp Gly Ile
1 5 6

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Asn Asp Asn Arg His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ile Met Asp Asp Gly Ile
1 5 6

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Trp Phe Asn Ser His
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCGGTTATCG ACGACGGTAT CGATTCT 27

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCGGTTATCG ACAAAGGTAT CGATTCT 27

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCGGTTATCG ACGAAGGTAT CGATTCT 27

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCAAGACAAC GACTCTCACG GAA 23

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCAAGACAAC AGCTCTCACG GAA 23

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCAAGACAAC AAATCTCACG GAA 23

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CACTTCCGGC AGCTCGTCGA CAGTGGACTA CCCTGGCAAA TA 42

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CACTTCCGGC AGCTCGTCGA CAGTGGAGTA CCCTGGCAAA TA 42

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTAACATGAG CCTCGGCCCA GCTAGCGGTT CTGCTGCTTT A 41

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TTAACATGAG CCTCGGCCCC GCGGATGATT CTGCTGCTTT AAA 43

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGGCAGCTCA AGCAACGATG GCTATCCTGG CAAATACCCT TCTGTCA 47

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ACTTCCGGCA GCTCTTCGAA CTACGACGGG TACCCTGGCA AATA 44

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Asn Leu Thr Ala Arg
1 5

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Asn Leu Met Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Thr Ala Ser Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Leu Thr Arg Arg Ser
1 5

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Ala Leu Ser Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Leu Met Leu Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Ala Ser Thr His Phe
1 5

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Gln Lys Pro Asn Phe
1 5

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Arg Lys Pro Thr His
1 5

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Ile Gln Gln Gln Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Arg Pro Gly Ala Met
1 5

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Gln Gly Glu Leu Pro
1 5

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Ala Pro Asp Pro Thr
1 5

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Gln Leu Leu Glu His
1 5

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Val Asn Asn Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Ala Gln Ser Asn Leu
1 5

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Thr Ala Ser Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

His His His His His His
1 5 6

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Leu Met Arg Lys
1 4

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Leu Thr Ala Arg
1 4

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Gly Pro Gly Gly
1 4

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gly Leu Met Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Ala Ala Pro Phe
1 4

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Gly Pro Gly Gly Xaa Xaa Xaa Xaa Xaa Gly Gly Pro Gly
1 5 10 13

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Ala Ala Pro Lys
1 4

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Ala Ala Pro Arg
1 4

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Ala Ala Pro Met
1 4

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Ala Ala Pro Gln
1 4

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Ala Ala Lys Phe
1 4

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Ala Ala Ala Phe
1 4

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Ala Ala Arg Phe
1 4

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Ala Ala Asp Phe

1 4

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Ala Ala Lys Lys
1 4

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Ala Ala Lys Arg
1 4

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Ala Ala Lys Phe
1 4

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Ala Ala Pro Xaa
1 4

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Ala Ala Xaa Phe
1 4

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Ala Ala Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 275 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala
1 5 10 15

Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val
20 25 30

Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala
35 40 45

Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp
50 55 60

Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu
65 70 75

Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu
80 85 90

Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser
95 100 105

Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp
110 115 120

Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu
125 130 135

Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val
140 145 150

Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val
155 160 165

Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val
170 175 180

Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro Glu
185 190 195

Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro
200 205 210

Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser Pro
215 220 225

His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
230 235 240

```
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr
                  245                 250                 255

Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val
                  260                 265                 270

Gln Ala Ala Ala Gln
                  275
```

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

```
Arg Val Arg Arg
 1           4
```

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1146 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

```
  GTG AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT 36
  Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe
-107   -105               -100

GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA 75
Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr
-95                 -90                 -85

TCC TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG 114
Ser Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys
        -80                 -75                 -70

AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG 153
Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
                -65                 -60

AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC 192
Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly
    -55                 -50                 -45

GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA GCT 231
Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala
            -40                 -35

TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA 270
Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
-30                 -25                 -20

AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT CAC GTA 309
Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val
        -15                 -10                 -5

AGA CAT AAG CGC GCG CAG TCC GTG CCT TAC GGC GTA TCA 348
```

```
Arg His Lys Arg Ala Gln Ser Val Pro Tyr Gly Val Ser
 1               5

CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA GGC TAC ACT 387
Gln Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr
 10                  15                  20

GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC 426
Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile
         25                  30                  35

GAT TCT TCT CAT CCT GAT TTA AAG GTA GCA GGC GGA GCC 465
Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
         40                  45

AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC 504
Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn
     50                  55                  60

GAC TCT CAC GGA ACT CAC GTT GCC GGC ACA GTT GCG GCT 543
Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala
         65                  70

CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC 582
Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser
 75                  80                  85

GCA TCA CTT TAC GCT GTA AAA GTT CTC GGT GCT GAC GGT 621
Ala Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly
         90                  95                  100

TCC GGC CAA GAT AGC TGG ATC ATT AAC GGA ATC GAG TGG 660
Ser Gly Gln Asp Ser Trp Ile Ile Asn Gly Ile Glu Trp
                 105                 110

GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC CTC 699
Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu
     115                 120                 125

GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT 738
Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val
             130                 135

GAT AAA GCC GTT GCA TCC GGC GTC GTA GTC GTT GCG GCA 777
Asp Lys Ala Val Ala Ser Gly Val Val Val Val Ala Ala
 140                 145                 150

GCC GGT AAC GAA GGC ACT TCC GGC AGC TCG TCG ACA GTG 816
Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val
         155                 160                 165

GAC TAC CCT GGC AAA TAC CCT TCT GTC ATT GCA GTA GGC 855
Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly
                 170                 175

GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC 894
Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser
     180                 185                 190

GTA GGA CCT GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT 933
Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser
             195                 200

ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC 972
Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr
```

```
205                 210                 215

AAC GGT ACC TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG 1011
Asn Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala
        220                 225                 230

GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC 1050
Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn
                235                 240

ACT CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA 1089
Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
    245                 250                 255

CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC 1128
Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
            260                 265

GTA CAG GCG GCA GCT CAG 1146
Val Gln Ala Ala Ala Gln
270                 275
```

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 382 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

```
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala
-107    -105                -100                -95

Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser Ser Ala Gln Ala
        -90                 -85                 -80

Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys
        -75                 -70                 -65

Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys Lys Asp Val Ile
        -60                 -55                 -50

Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp
        -45                 -40                 -35

Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
        -30                 -25                 -20

Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Arg His
        -15                 -10                  -5

Lys Arg Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala
          1                   5                  10

Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val
     15                  20                  25

Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys
     30                  35                  40

Val Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe
     45                  50                  55

Gln Asp Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala
```

60 65 70

Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala
75 80 85

Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln
90 95 100

Asp Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn
105 110 115

Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala
120 125 130

Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val
135 140 145

Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser
150 155 160

Thr Val Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly
165 170 175

Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly
180 185 190

Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
195 200 205

Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala
210 215 220

Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His
225 230 235

Pro Asn Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr
240 245 250

Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile
255 260 265

Asn Val Gln Ala Ala Ala Gln
270 275

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Asn Arg Met Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

Gly Asp Ile Thr Thr Glu Asp Glu Ala Ala Ser
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Gly Glu Val Thr Asp Ala Val Glu Ala Arg Ser
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Pro Phe Met Thr Asp Ile Ile Glu Ala Ser Ser
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Gly Ile Val Thr Asp Ala Ile Glu Ala Ser Ser
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GGTTCCGGCC AAGATAGCTG GATCATT 27

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCAATACAGC TGGGAAATTA ACGGAATCG 29

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

GGTTCCGGCC AAGATAGCTG GGAAATTAAC G 31

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

AAGAAGATCA CGTAAGACAT AAGCGCGCGC 30

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Arg Ala Lys Arg
1 4

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Lys Ala Lys Arg
1 4

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids

(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

Gly Pro Gly Gly Leu Met Arg Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:89:

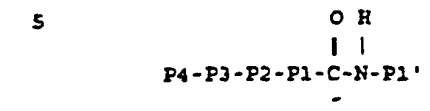
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Gly Pro Gly Gly Lys Ala Lys Arg
1 5 8

What is claimed is:

1. A subtilisin variant derived from a precursor subtilisin-type serine protease said variant capable of cleaving a polypeptide substrate comprising the sequence:



wherein;

P4 is a basic amino acid;

P3 is any amino acid selected from the naturally occurring amino acids;

P2 is a basic amino acid;

P1 is a basic amino acid; and

P1' is not Pro.

2. The subtilisin variant of claim 1 containing an acidic amino acid at a residue equivalent to Asn 62, Tyr 104 and Gly 166 of the subtilisin naturally produced by *Bacillus amyloliquefaciens*.

3. The subtilisin-type serine protease variant of claim 2 wherein the acidic amino acid is Asp or Glu.

4. The subtilisin-type serine protease variant of claim 3 wherein the acidic amino acid is Asp.

5. The subtilisin-type serine protease variant of claim 2 wherein the precursor subtilisin-type serine protease in the subtilisin naturally produced by *Bacillus amyloliquefaciens*.

6. The subtilisin variant of claim 5 having the amino acid sequence of the mature polypeptide of Figure 8 (SEQ ID NO: 75).

7. A subtilisin variant having substrate specificity for peptide substrates containing dibasic amino acid sequences.

8. The subtilisin variant of claim 7 having a different amino acid residue at residue position +62 than subtilisin naturally produced by *Bacillus amyloliquefaciens*.

9. The subtilisin variant of Claim 8 having an Asp or Glu at residue position +62.

10. The subtilisin variant of Claim 9 having an Asp at residue position +62.

11. The subtilisin variant of Claim 10 further having an Asp or Glu at residue position +166.

12. The subtilisin variant of Claim 11 having an Asp at residue position +166.

13. The subtilisin variant of Claim 12 having the amino acid sequence of the mature polypeptide provided in Fig. 6.

14. An isolated nucleic acid molecule encoding the subtilisin variant of Claim 1.

15. The nucleic acid molecule of Claim 14 further comprising a promoter operably linked to the nucleic acid molecule.

16. An expression vector comprising the nucleic acid molecule of Claim 15 operably linked to control sequences recognized by a host cell transformed with the vector.

17. A host cell transformed with the vector of Claim 16.

18. An isolated nucleic acid molecule encoding the subtilisin variant of Claim 7.

19. The nucleic acid molecule of Claim 18 further comprising a promoter operably linked to the nucleic acid molecule.

20. An expression vector comprising the nucleic acid molecule of Claim 19 operably linked to control sequences recognized by a host cell transformed with the vector.

21. A host cell transformed with the vector of Claim 20.

22. A process of using the nucleic acid molecule encoding the subtilisin variant to effect production of the subtilisin variant comprising culturing the host cell of Claim 21 under conditions suitable for expression of the subtilisin variant.

23. The process of Claim 22 further comprising recovering the subtilisin variant from the host cell culture medium.

24. A method of using the subtilisin variant of Claim 1 comprising contacting a fusion protein containing a dibasic sequence with the subtilisin variant.

25. A process for cleaving a polypeptide, said polypeptide comprising an amino acid sequence represented by the formula:

P4-P3-P2-P1-P1'

wherein,

P4 is a basic amino acid;

P3 is an amino acid selected from the naturally occurring amino acids;

P2 is a basic amino acid;

P1 is a basic amino acid; and

P1' is not Pro;

comprising the step of:

subjecting said polypeptide to the subtilisn variant of claim 1 in a reaction mixture under conditions such that the subtilisn variant cleaves the polypeptide.

26. A process of using the nucleic acid molecule encoding the subtilisin variant to effect production of the subtilisin variant comprising culturing the host cell of Claim 17 under conditions suitable for expression of the subtilisin variant.

27. The process of Claim 26 further comprising recovering the subtilisin variant from the host cell culture medium.

28. A method of using the subtilisin variant of Claim 7 comprising contacting a fusion protein containing a dibasic sequence with the subtilisin variant.

29. A process for cleaving a polypeptide, said polypeptide comprising an amino acid sequence represented by the formula:

P4-P3-P2-P1-P1'

wherein,

P4 is a large hydrophobic amino acid;

P3 is an amino acid selected from the naturally occurring amino acids;

P2 is a basic amino acid;

P1 is a basic amino acid; and

P1' is not Pro;

comprising the step of:

subjecting said polypeptide to the subtilisn variant of claim 7 in a reaction mixture under conditions such that the subtilisn variant cleaves the polypeptide.

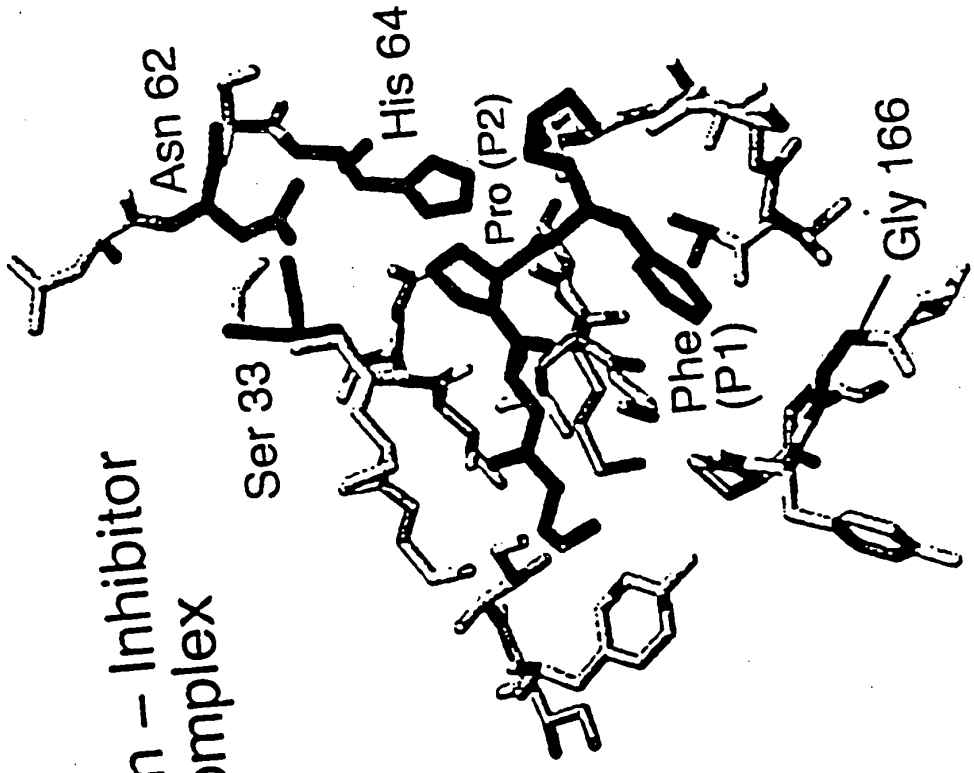

Subtilisin – Inhibitor Complex
Asn 62
His 64
Ser 33
Pro (P2)
Phe (P1)
Gly 166

Figure 1

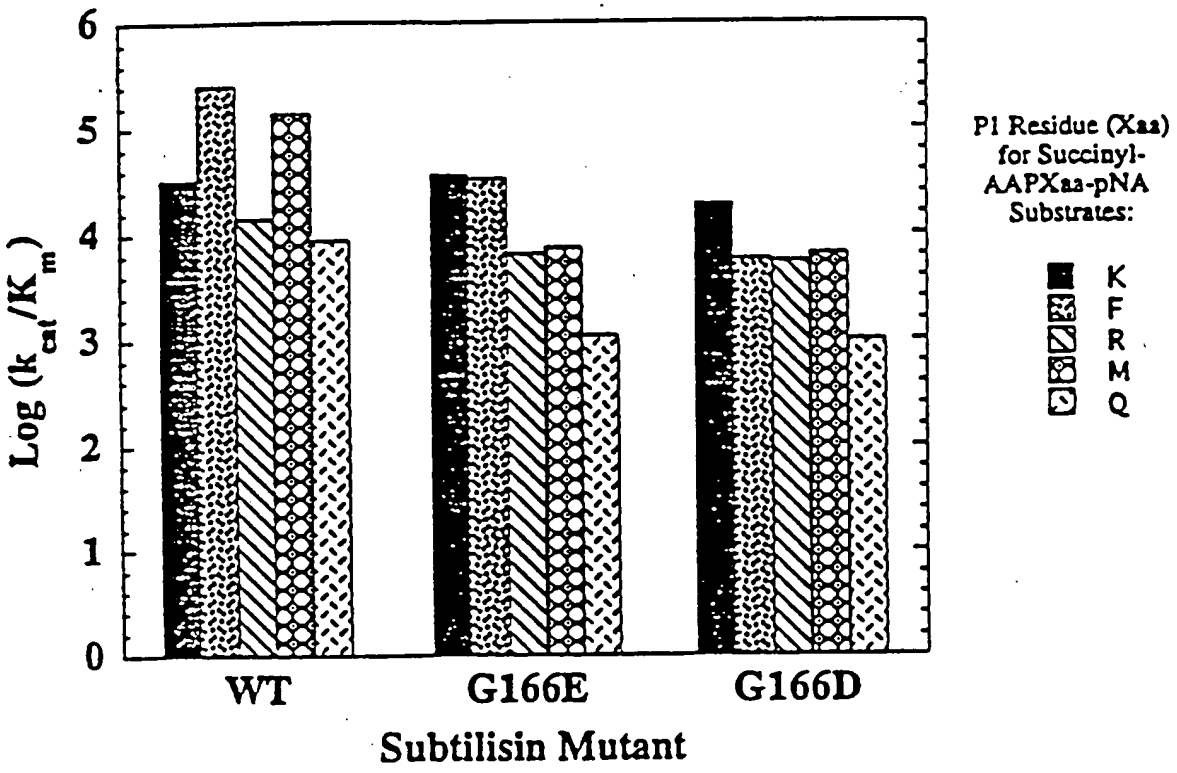

6
5
4
3
2
1
0
Log (k_{cat}/K_m)
WT
G166E
G166D
Subtilisin Mutant
P1 Residue (Xaa) for Succinyl-AAPXaa-pNA Substrates:
K
F
R
M
Q

Figure 2

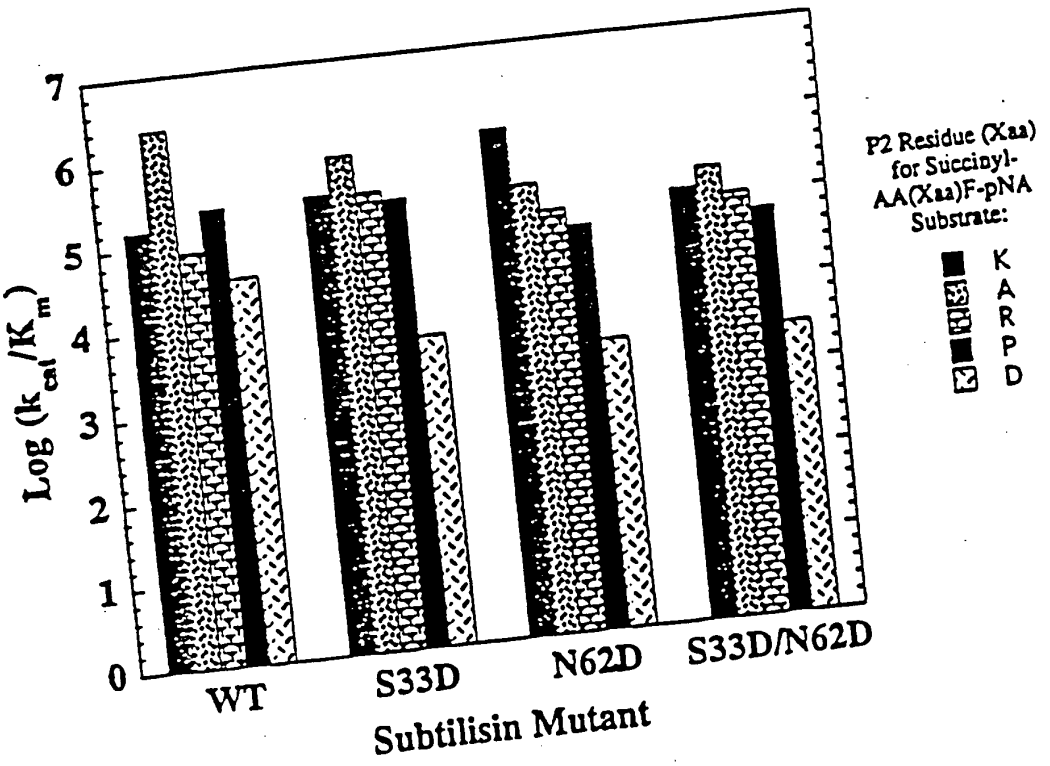

Log (k_{cat}/K_m)
0
1
2
3
4
5
6
7
WT
S33D
N62D
S33D/N62D
Subtilisin Mutant
P2 Residue (Xaa) for Succinyl-AA(Xaa)F-pNA Substrate:
K
A
R
P
D

Figure 3

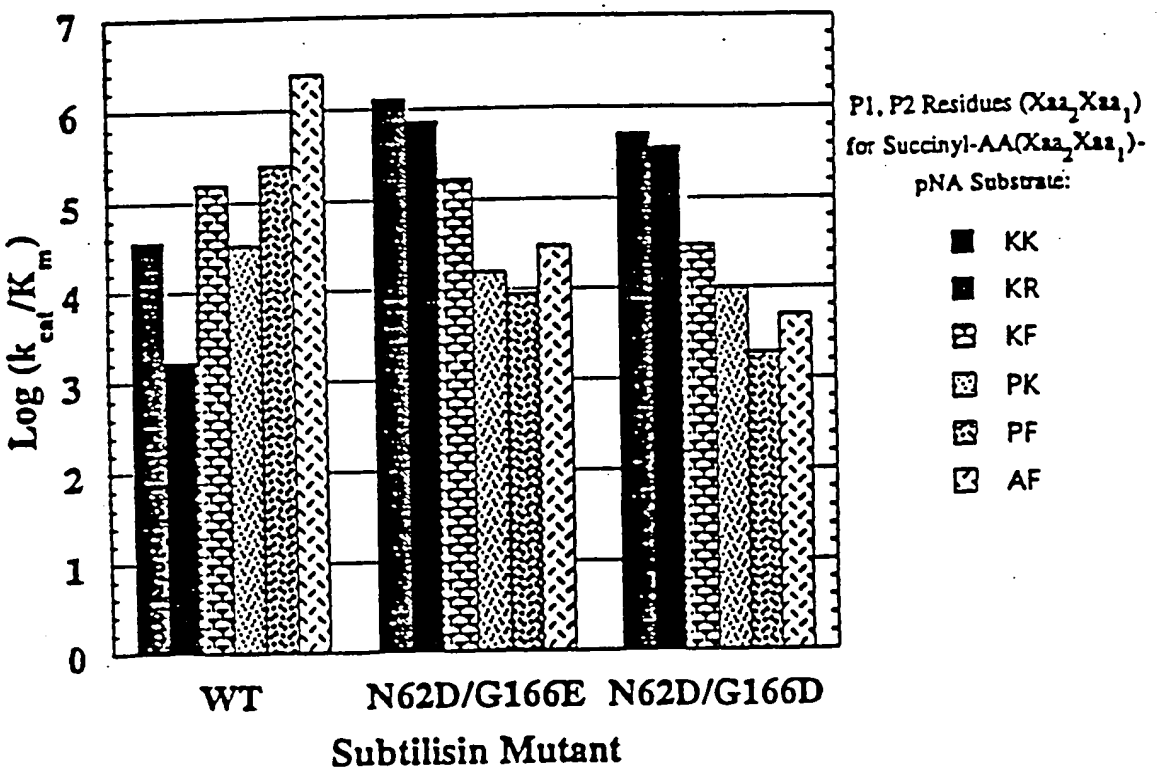

7
6
5
4
3
2
1
0
Log (k_{cat}/K_m)
WT
N62D/G166E
N62D/G166D
Subtilisin Mutant
P1, P2 Residues (Xaa_2Xaa_1) for Succinyl-AA(Xaa_2Xaa_1)-pNA Substrate:
KK
KR
KF
PK
PF
AF

Figure 4

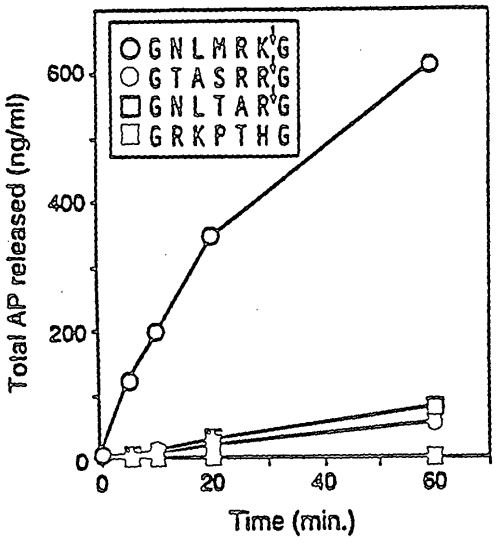

GNLMRK↓G
GTASRR↓G
GNLTAR↓G
GRKPTHG
600
400
200
0
0
20
40
60
Total AP released (ng/ml)
Time (min.)

FIGURE 5

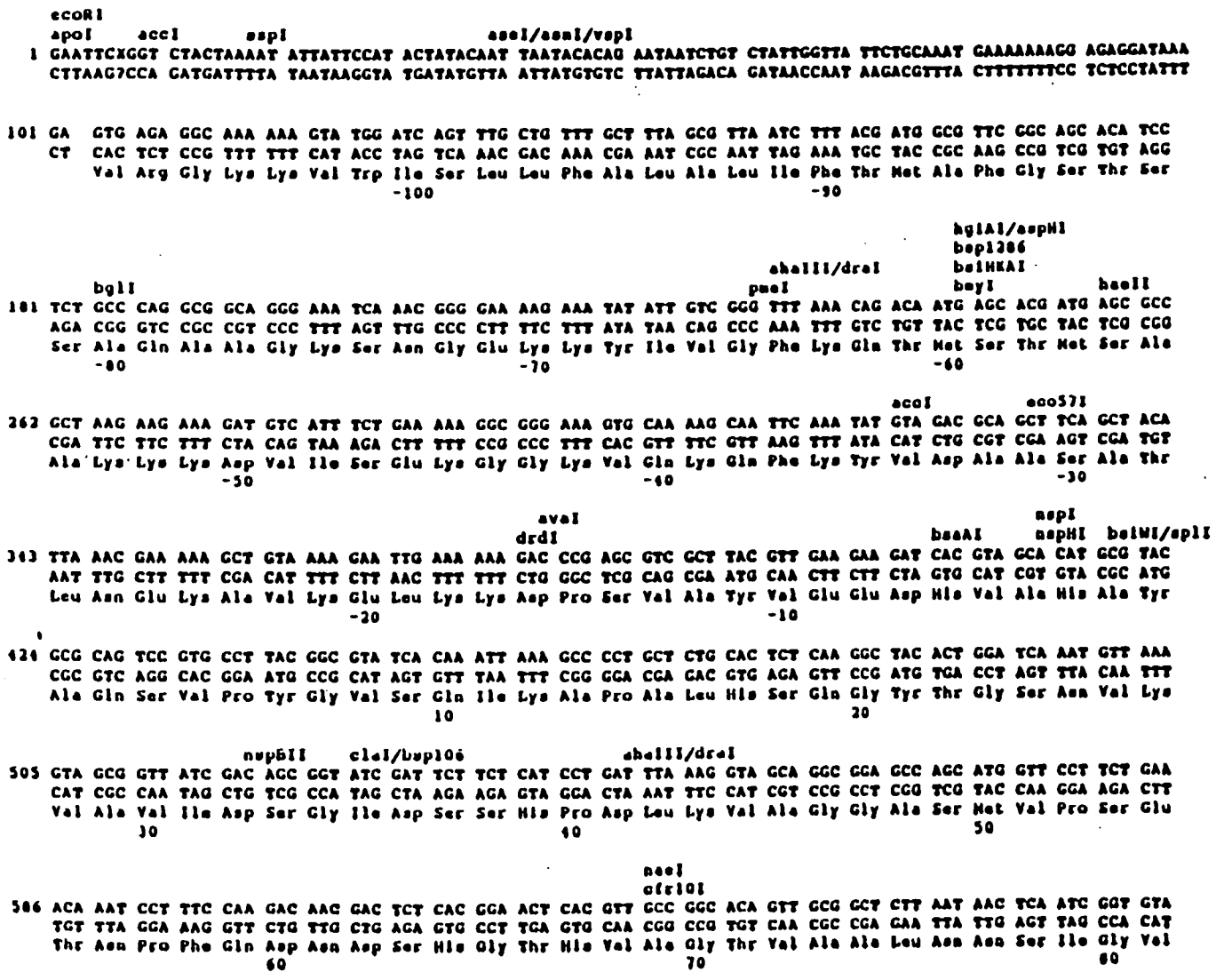

ecoRI
apoI
accI
sspI
aseI/asnI/vspI
1 GAATTCNGGT CTACTAAAAT ATTATTCCAT ACTATACAAT TAATACACAG AATAATCTGT CTATTGGTTA TTCTGCAAAT GAAAAAAAGG AGAGGATAAA
CTTAAG?CCA GATGATTTTA TAATAAGGTA TGATATGTTA ATTATGTGTC TTATTAGACA GATAACCAAT AAGACGTTTA CTTTTTTTCC TCTCCTATTT
101 GA GTG AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA TCC
CT CAC TCT CCG TTT TTT CAT ACC TAG TCA AAC GAC AAA CGA AAT CGC AAT TAG AAA TGC TAC CGC AAG CCG TCG TGT AGG
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
-100 -90
hglAI/aspHI
bsp1286
ahaIII/draI
bsiHKAI
bglI
pmeI
beyI
haeII
181 TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG AGC GCC
AGA CGG GTC CGC CGT CCC TTT AGT TTG CCC CTT TTC TTT ATA TAA CAG CCC AAA TTT GTC TGT TAC TCG TGC TAC TCG CGG
Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met Ser Ala
-80 -70 -60
acoI
acc571
262 GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA GCT TCA GCT ACA
CGA TTC TTC TTT CTA CAG TAA AGA CTT TTT CCG CCC TTT CAC GTT TTC GTT AAG TTT ATA CAT CTG CGT CGA AGT CGA TGT
Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala Ser Ala Thr
-50 -40 -30
aspI
aval
drdI
bssAI
aspHI
bsiWI/splI
343 TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT CAC GTA GCA CAT GCG TAC
AAT TTG CTT TTT CGA CAT TTT CTT AAC TTT TTT CTG GGC TCG CAG CGA ATG CAA CTT CTT CTA GTG CAT CGT GTA CGC ATG
Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Ala His Ala Tyr
-20 -10
424 GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA GGC TAC ACT GGA TCA AAT GTT AAA
CGC GTC AGG CAC GGA ATG CCG CAT AGT GTT TAA TTT CGG GGA CGA GAC GTG AGA GTT CCG ATG TGA CCT AGT TTA CAA TTT
Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys
10 20
nsp6II
claI/bsp106
ahaIII/draI
505 GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA GCA GGC GGA GCC AGC ATG GTT CCT TCT GAA
CAT CGC CAA TAG CTG TCG CCA TAG CTA AGA AGA GTA GGA CTA AAT TTC CAT CGT CCG CCT CGG TCG TAC CAA GGA AGA CTT
Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala Ser Met Val Pro Ser Glu
30 40 50
haeI
afrI0I
586 ACA AAT CCT TTC CAA GAC AAC GAC TCT CAC GGA ACT CAC GTT GCC GGC ACA GTT GCG GCT CTT AAT AAC TCA ATC GGT GTA
TGT TTA GGA AAG GTT CTG TTG CTG AGA GTG CCT TGA GTG CAA CGG CCG TGT CAA CGC CGA GAA TTA TTG AGT TAG CCA CAT
Thr Asn Pro Phe Gln Asp Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly Val
60 70 80

Figure 6-1

```
                                                                                eaeI        pvuII
                                                                                cfrI        nspBII
667  TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT
     AAT CCG CAA CGC GGT TCG CGT AGT GAA ATG CGA CAT TTT CAA GAG CCA CGA CTG CCA AGG CCG GTT ATG TCG ACC TAG TAA
     Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile
                                     90                                     100

                      pvuI/bspCI
                      mcrI                                                                                ahaIII/draI
748  AAC GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA
     TTG CCT TAG CTC ACC CGC TAG CGT TTG TTA TAC CTG CAA TAA TTG TAC TCG GAG CCG CCT GGA AGA CCA AGA CGA CGA AAT
     Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu
         110                                 120                                     130

                                                                                                          salI
                                         hinlI/acyI                                                       hincII/hindII
                                         ahaII/bsaHI                  cfr10I                              accI
829  AAA GCG GCA GTT GAT AAA GCC GTT GCA TCC GGC GTC GTA GTC GTT GCG GCA GCC GGT AAC GAA GGC ACT TCC GGC AGC TCG
     TTT CGC CGT CAA CTA TTT CGG CAA CGT AGG CCG CAG CAT CAG CAA CGC CGT CGG CCA TTG CTT CCG TGA AGG CCG TCG AGC
     Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser
                     140                                 150                                     160

                                                                  haeII    hincII/hindII
910  TCG ACA GTG GAC TAC CCT GGC AAA TAC CCT TCT GTC ATT GCA GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC
     AGC TGT CAC CTG ATG GGA CCG TTT ATG GGA AGA CAG TAA CGT CAT CCG CGA CAA CTG TCG TCG TTG GTT TCT CGT AGA AAG
     Ser Thr Val Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe
                                 170                                    180

             ppuMI                           hgiCI
             eco0109I/draII                  banI
991  TCA AGC GTA GGA CCT GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG
     AGT TCG CAT CCT GGA CTC GAA CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT TCG TGC GAA GGA CCT TTG TTT ATG CCC CGC
     Ser Ser Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala
     190                                     200                                     210

             kpnI
             hgiCI
             banI
             asp718
             acc65I                                   bglI bsrBI
1073 TAC AAC GGT ACC TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC
     ATG TTG CCA TGG AGT TAC CGT AGA GGC GTG CAA CGG CCT CGC CGA CGA AAC TAA GAA AGA TTC GTG GGC TTG ACC TGT TTG
     Tyr Asn Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn
                 220                                     230                                     240

                                                                                                  bclI(dam-)
1153 ACT CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC GTA
     TGA GTT CAG GCG TCG TCA AAT CTT TTG TGG TGA TGT TTT GAA CCA CTA AGA AAG ATG ATA CCT TTT CCC GAC TAG TTG CAT
     Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val
                             250                                     260                                     270
```

Figure 6-2

```
                                    styI                                      nspI
                                cfr10I          cfr10I                        nspHI        bsrBI
1234 CAG GCG GCA GCT CAG TAA AACATAAAA AACCGGCCTT GGCCCCGCCG GTTTTTTATT ATTTTTCTTC CTCCGCATGT TCAATCCGCT
     GTC CGC CGT CGA GTC ATT TTGTATTTT TTGGCCGGAA CCGGGGCGGC CAAAAAATAA TAAAAAGAAG GAGGCGTACA AGTTAGGCGA
     Gln Ala Ala Ala Gln Oc*

                                                                             eaeI
                                 apoI                  hincII/hindII         cfrI        esp3I
1321 CCATAATCGA CGGATGGCTC CCTCTGAAAA TTTTAACGAG AAACGGCGGG TTGACCCGGC TCAGTCCCGT AACGGCCAAG TCCTGAAACG TCTCAATCGC
     GGTATTAGCT GCCTACCGAG GGAGACTTTT AAAATTGCTC TTTGCCGCCC AACTGGGCCG AGTCAGGGCA TTGCCGGTTC AGGACTTTGC AGAGTTAGCG

                                                                                          aroI
                                                                                          bspHII
                                                                                          bspEI(dam-)
                                                                                          bsaWI
                                                                                          acoIII(dam-)
                                                                                        bstYI/xhoII
                                                                          esp3I bsmI   bamHI          psp1406I
                 bsaWI               mcrI
1421 CGCTTCCCGG TTTCCGGTCA GCTCAATGCC GTAACGGTCG GCGGCGTTTT CCTGATACCG GGAGACGGCA TTCGTAATCG GATCCGGAAA TTGTAAACGT
     GCGAAGGGCC AAAGGCCAGT CGAGTTACGG CATTGCCAGC CGCCGCAAAA GGACTATGGC CCTCTGCCGT AAGCATTAGC CTAGGCCTTT AACATTTGCA

      sspI        apoI        apoI
1521 TAATATTTTG TTAAAATTCG CGTTAAATTT TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC GGCAAAATCC CTTATAAATC AAAAGAATAG
     ATTATAAAAC AATTTTAAGC GCAATTTAAA AACAATTTAG TCGAGTAAAA AATTGGTTAT CCGGCTTTAG CCGTTTTAGG GAATATTTAG TTTTCTTATC

                                                                      drdI
1621 ACCGAGATAG GGTTGAGTGT TGTTCCAGTT TGGAACAAGA GTCCACTATT AAAGAACGTG GACTCCAACG TCAAAGGGCG AAAAACCGTC TATCAGGGCT
     TGGCTCTATC CCAACTCACA ACAAGGTCAA ACCTTGTTCT CAGGTGATAA TTTCTTGCAC CTGAGGTTGC AGTTTCCCGC TTTTTGGCAG ATAGTCCCGA

                                                                                         hgiJII
                                                                                         bsp1286
                                                                                         bmyI
                                                bglCI                                    banII
             bsaAI                              banI
          draIII
1721 ATGGCCCACT ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG GAACCCTAAA GGGAGCCCCC GATTTAGAGC
     TACCGGGTGA TGCACTTGGT AGTGGGATTA GTTCAAAAAA CCCCAGCTCC ACGGCATTTC GTGATTTAGC CTTGGGATTT CCCTCGGGGG CTAAATCTCG

                                                            haeII
              naeI                                  bsrBI           haeII
              cfr10I
1821 TTGACGGGGA AAGCCGGCGA ACGTGGCGAG AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG CTGGCAAGTG TAGCGGTCAC GCTGCGCGTA
     AACTGCCCCT TTCGGCCGCT TGCACCGCTC TTTCCTTCCC TTCTTTCGCT TTCCTCGCCC GCGATCCCGC GACCGTTCAC ATCGCCAGTG CGACGCGCAT

                                                aroI
                                                bspHII
                                                bspEI(dam-)
                                                bsaWI
                                     sofI       acoIII(dam-)                haeI
1921 ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCCGGATC XGATCCGACG CGAGGCTGGA TGGCCTTCCC CATTATGATT CTTCTCGCTT
     TGGTGGTGTG GGCGGCGCGA ATTACGCGGC GATGTCCCGC GCAGGCCTAG ?CTAGGCTGC GCTCCGACCT ACCGGAAGGG GTAATACTAA GAAGAGCGAA
```

Figure 6-3

Figure 6-4

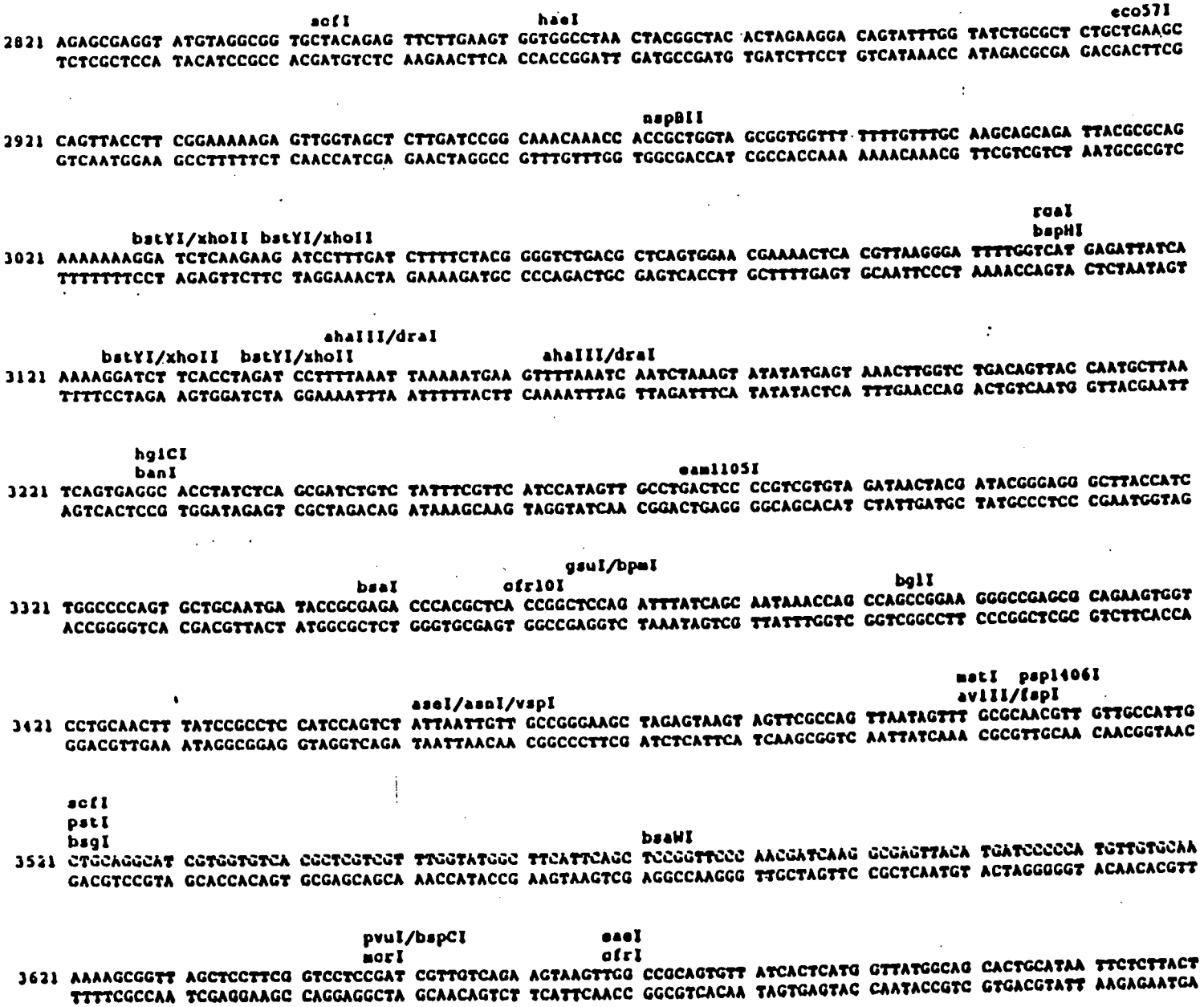

scfI
haeI
eco57I
2821 AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG TATCTGCGCT CTGCTGAAGC
TCTCGCTCCA TACATCCGCC ACGATGTCTC AAGAACTTCA CCACCGGATT GATGCCGATG TGATCTTCCT GTCATAAACC ATAGACGCGA GACGACTTCG
nspBII
2921 CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC AAGCAGCAGA TTACGCGCAG
GTCAATGGAA GCCTTTTTCT CAACCATCGA GAACTAGGCC GTTTGTTTGG TGGCGACCAT CGCCACCAAA AAAACAAACG TTCGTCGTCT AATGCGCGTC
rcaI
bspHI
bstYI/xhoII bstYI/xhoII
3021 AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA
TTTTTTTCCT AGAGTTCTTC TAGGAAACTA GAAAAGATGC CCCAGACTGC GAGTCACCTT GCTTTTGAGT GCAATTCCCT AAAACCAGTA CTCTAATAGT
ahaIII/draI
bstYI/xhoII bstYI/xhoII
ahaIII/draI
3121 AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAACTTGGTC TGACAGTTAC CAATGCTTAA
TTTTCCTAGA AGTGGATCTA GGAAAATTTA ATTTTTACTT CAAAATTTAG TTAGATTTCA TATATACTCA TTTGAACCAG ACTGTCAATG GTTACGAATT
hgiCI
banI
eam1105I
3221 TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC
AGTCACTCCG TGGATAGAGT CGCTAGACAG ATAAAGCAAG TAGGTATCAA CGGACTGAGG GGCAGCACAT CTATTGATGC TATGCCCTCC CGAATGGTAG
gsuI/bpmI
bsaI
afr10I
bglI
3321 TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT
ACCGGGGTCA CGACGTTACT ATGGCGCTCT GGGTGCGAGT GGCCGAGGTC TAAATAGTCG TTATTTGGTC GGTCGGCCTT CCCGGCTCGC GTCTTCACCA
mstI psp1406I
avlII/fspI
aseI/asnI/vspI
3421 CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG
GGACGTTGAA ATAGGCGGAG GTAGGTCAGA TAATTAACAA CGGCCCTTCG ATCTCATTCA TCAAGCGGTC AATTATCAAA CGCGTTGCAA CAACGGTAAC
scfI
pstI
bsgI
bsaWI
3521 CTGCAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA TGATCCCCCA TGTTGTGCAA
GACGTCCGTA GCACCACAGT GCGAGCAGCA AACCATACCG AAGTAAGTCG AGGCCAAGGG TTGCTAGTTC CGCTCAATGT ACTAGGGGGT ACAACACGTT
pvuI/bspCI
eaeI
morI
afrI
3621 AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT
TTTTCGCCAA TCGAGGAAGC CAGGAGGCTA GCAACAGTCT TCATTCAACC GGCGTCACAA TAGTGAGTAC CAATACCGTC GTGACGTATT AAGAGAATGA

Figure 6-5

```
                                                                               hincII/hindII
                                                          mcrI                  hinII/acyI
                                       scaI               bsgI  |              ahaII/bsaHI
3721 GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC GAGTTGCTCT TGCCCGGCGT
     CAGTACGGTA GGCATTCTAC GAAAAGACAC TGACCACTCA TGAGTTGGTT CAGTAAGACT CTTATCACAT ACGCCGCTGG CTCAACGAGA ACGGGCCGCA

                                       hgiAI/aspHI
                                       bsp1286
                                       bsiHKAI       psp1406I
                                       bsyI       xmnI                                     aspBII
                                  ahaIII/draI     asp700                          bstYI/xhoII
3821 CAACACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC TCAAGGATCT TACCGCTGTT
     GTTGTGCCCT ATTATGGCGC GGTGTATCGT CTTGAAATTT TCACGAGTAG TAACCTTTTG CAAGAAGCCC CGCTTTTGAG AGTTCCTAGA ATGGCGACAA

                       hgiAI/aspHI
                       bsp1286
                       bsiHKAI
                       bsyI
                       apaLI/snoI
 bstYI/xhoII           alw44I/snoI  eco57I
3921 GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT
     CTCTAGGTCA AGCTACATTG GGTGAGCACG TGGGTTGACT AGAAGTCGTA GAAAATGAAA GTGGTCGCAA AGACCCACTC GTTTTTGTCC TTCCGTTTTA

                                                                                                bsrBI
                                                                                             rcaI
                                               earI/ksp632I  sspI                            bspHI
4021 GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA
     CGGCGTTTTT TCCCTTATTC CCGCTGTGCC TTTACAACTT ATGAGTATGA GAAGGAAAAA GTTATAATAA CTTCGTAAAT AGTCCCAATA ACAGAGTACT

                                                                          hinII/acyI
                                                                          ahaII/bsaHI         rcaI
                                                                          aatII               bspHI
4121 GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC GTCTAAGAAA CCATTATTAT
     CGCCTATGTA TAAACTTACA TAAATCTTTT TATTTGTTTA TCCCCAAGGC GCGTGTAAAG GGGCTTTTCA CGGTGGACTG CAGATTCTTT GGTAATAATA

                                          bpuAI     asnI/aseI/vspI                                  psp1406I
                                          bbsI     xmnI                                        ahaIII/draI
                                  eco0109I/draII   asp700     aflII/bfrI
4221 CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC TTTCGTCTTC AAGAATTAAT TCCTTAAGCA ACGTACAGAC GGCTTAAAAG CCTTTAAAAA
     GTACTGTAAT TGGATATTTT TATCCGCATA GTGCTCCGGG AAAGCAGAAG TTCTTAATTA AGGAATTCGT TGCATGTCTG CCGAATTTTC GGAAATTTTT

                asoI                                                            dpoI
4321 CGTTTTTAAG GGGTTTGTAG ACAAGGTAAA GGATAAAACA GCACAATTCC AAGAAAAACA CGATTTAGAA CCTAAAAAGA ACGAATTTGA ACTAACTCAT
     GCAAAAATTC CCCAAACATC TGTTCCATTT CCTATTTTGT CGTGTTAAGG TTCTTTTTGT GCTAAATCTT GGATTTTTCT TGCTTAAACT TGATTGAGTA
```

Figure 6-6

```
                                                                                                          xmnI
                                                                                                          asp700
4421 AACCGAGAGG TAAAAAAAGA ACGAAGTCGA GATCAGGGAA TGAGTTTATA AAATAAAAAA AGCACCTGAA AAGGTGTCTT TTTTTGATGG TTTTGAACTT
     TTGGCTCTCC ATTTTTTTCT TGCTTCAGCT CTAGTCCCTT ACTCAAATAT TTTATTTTTT TCGTGGACTT TTCCACAGAA AAAAACTACC AAAACTTGAA

                                                                                                    ahaIII/draI
4521 GTTCTTTCTT ATCTTGATAC ATATAGAAAT AACGTCATTT TTATTTTAGT TGCTGAAAGG TGCGTTGAAG TGTTGGTATG TATGTGTTTT AAAGTATTGA
     CAAGAAAGAA TAGAACTATG TATATCTTTA TTGCAGTAAA AATAAAATCA ACGACTTTCC ACGCAACTTC ACAACCATAC ATACACAAAA TTTCATAACT

                                                                                                      sspI
4621 AAACCCTTAA AATTGGTTGC ACAGAAAAAC CCCATCTGTT AAAGTTATAA GTGACTAAAC AAATAACTAA ATAGATGGGG GTTTCTTTTA ATATTATGTG
     TTTGGGAATT TTAACCAACG TGTCTTTTTG GGGTAGACAA TTTCAATATT CACTGATTTG TTTATTGATT TATCTACCCC CAAAGAAAAT TATAATACAC

                                                                               styI
                                                                               ncoI
                                                                          bsaI dsaI
4721 TCCTAATAGT AGCATTTATT CAGATGAAAA ATCAAGGGTT TTAGTGGACA AGACAAAAAG TGGAAAAGTG AGACCATGGA GAGAAAAGAA AATCGCTAAT
     AGGATTATCA TCGTAAATAA GTCTACTTTT TAGTTCCCAA AATCACCTGT TCTGTTTTTC ACCTTTTCAC TCTGGTACCT CTCTTTTCTT TTAGCGATTA

                                     ahaIII/draI
                                  apoI                                    sspI
4821 GTTGATTACT TTGAACTTCT GCATATTCTT GAATTTAAAA AGGCTGAAAG AGTAAAAGAT TGTGCTGAAA TATTAGAGTA TAAACAAAAT CGTGAAACAG
     CAACTAATGA AACTTGAAGA CGTATAAGAA CTTAAATTTT TCCGACTTTC TCATTTTCTA ACACGACTTT ATAATCTCAT ATTTGTTTTA GCACTTTGTC

                                                  xcmI         gsuI/bpmI                bsmI
4921 GCGAAAGAAA GTTGTATCGA GTGTGGTTTT GTAAATCCAG GCTTTGTCCA ATGTGCAACT GGAGGAGAGC AATGAAACAT GGCATTCAGT CACAAAAGGT
     CGCTTTCTTT CAACATAGCT CACACCAAAA CATTTAGGTC CGAAACAGGT TACACGTTGA CCTCCTCTCG TTACTTTGTA CCGTAAGTCA GTGTTTTCCA

          eco57I                 xcmI
5021 TGTTGCTGAA GTTATTAAAC AAAAGCCAAC AGTTCGTTGG TTGTTTCTCA CATTAACAGT TAAAAATGTT TATGATGGCG AAGAATTAAA TAAGAGTTTG
     ACAACGACTT CAATAATTTG TTTTCGGTTG TCAAGCAACC AACAAAGAGT GTAATTGTCA ATTTTTACAA ATACTACCGC TTCTTAATTT ATTCTCAAAC

                                                     aseI/asnI/vspI
5121 TCAGATATGG CTCAAGGATT TCGCCGAATG ATGCAATATA AAAAAATTAA TAAAAATCTT GTTGGTTTTA TGCGTGCAAC GGAAGTGACA ATAAATAATA
     AGTCTATACC GAGTTCCTAA AGCGGCTTAC TACGTTATAT TTTTTTAATT ATTTTTAGAA CAACCAAAAT ACGCACGTTG CCTTCACTGT TATTTATTAT

                               nspI
                               nspHI
                              ppu10I
                              nsiI/avaIII
                             nspI
                             nspHI                                                     bsaAI
5221 AAGATAATTC TTATAATCAG CACATGCATG TATTGGTATG TGTGGAACCA ACTTATTTTA AGAATACAGA AAACTACGTG AATCAAAAAC AATGGATTCA
     TTCTATTAAG AATATTAGTC GTGTACGTAC ATAACCATAC ACACCTTGGT TGAATAAAAT TCTTATGTCT TTTGATGCAC TTAGTTTTTG TTACCTAAGT
```

Figure 6-7

```
                                                         asuII                                         aseI
5331 ATTTTGGAAA AAGGCAATGA AATTAGACTA TGATCCAAAT GTAAAAGTTC AAATGATTCG ACCGAAAAAT AAATATAAAT CGGATATACA ATCGGCAATT
     TAAAACCTTT TTCCGTTACT TTAATCTGAT ACTAGGTTTA CATTTTCAAG TTTACTAAGC TGGCTTTTTA TTTATATTTA GCCTATATGT TAGCCGTTAA

                                                         apoI       psp1406I
5431 GACGAAACTG CAAAATATCC TGTAAAGGAT ACGGATTTTA TGACCGATGA TGAAGAAAAG AATTTGAAAC GTTTGTCTGA TTTGGAGCAA GGTTTACACC
     CTGCTTTGAC GTTTTATAGG ACATTTCCTA TGCCTAAAAT ACTGGCTACT ACTTCTTTTC TTAAACTTTG CAAACAGACT AAACCTCGTT CCAAATGTGG

5531 GTAAAAGGTT AATCTCCTAT GGTGGTTTGT TAAAAGAAAT ACATAAAAAA TTAAACCTTG ATGACACAGA AGAAGGCGAT TTGATTCATA CAGATGATGA
     CATTTTCCAA TTAGAGGATA CCACCAAACA ATTTTCTTTA TGTATTTTTT AATTTGGAAC TACTGTGTCT TCTTCCGCTA AACTAAGTAT GTCTACTACT

5631 CGAAAAAGCC GATGAAGATG GATTTTCTAT TATTGCAATG TGGAATTGGG AACGGAAAAA TTATTTTATT AAAGAGTAGT TCAACAAACG GGCCAGTTTG
     GCTTTTTCGG CTACTTCTAC CTAAAAGATA ATAACGTTAC ACCTTAACCC TTGCCTTTTT AATAAAATAA TTTCTCATCA AGTTGTTTGC CCGGTCAAAC

                                                                                                hgiAI/aspHI
                                                                                                bsp1286
                                                                                                bsiHKAI
                                                    bpuAI                                       bmyI       sspI
                                                    bbsI       aseI/asnI/vspI
5731 TTGAAGATTA GATGCTATAA TTGTTATTAA AAGGATTGAA GGATGCTTAG GAAGACGAGT TATTAATAGC TGAATAAGAA CGGTGCTCTC CAAATATTCT
     AACTTCTAAT CTACGATATT AACAATAATT TTCCTAACTT CCTACGAATC CTTCTGCTCA ATAATTATCG ACTTATTCTT GCCACGAGAG GTTTATAAGA

                                                                                                           nspI
                                                                                                           bspHI
5831 TATTTAGAAA AGCAAATCTA AAATTATCTG AAAAGGGAAT GAGAATAGTG AATGGACCAA TAATAATGAC TAGAGAAGAA AGAATGAAGA TTGTTCATGA
     ATAAATCTTT TCGTTTAGAT TTTAATAGAC TTTTCCCTTA CTCTTATCAC TTACCTGGTT ATTATTACTG ATCTCTTCTT TCTTACTTCT AACAAGTACT

                                                                                                hgiJII
                                                                                                eco0109I/draII
                                                                                                bsp1286
                                                                                                bsp120I
                                                                                                bmyI
                                                                                                banII
           sspI                                                                                 apaI
5931 AATTAAGGAA CGAATATTGG ATAAATATGG GGATGATGTT AAGGCTATTG GTGTTTATGG CTCTCTTGGT CGTCAGACTG ATGGGCCCTA TTCGGATATT
     TTAATTCCTT GCTTATAACC TATTTATACC CCTACTACAA TTCCGATAAC CACAAATACC GAGAGAACCA GCAGTCTGAC TACCCGGGAT AAGCCTATAA

                                                  cfr10I                                                   asnI
                                                  bsaWI                                                    aseI/bsp63II
                               hincII/hindII      agoI                          apoI                       asp700
6031 GAGATGATGT GTGTCATGTC AACAGAGGAA GCAGAGTTCA GCCATGAATG GACAACCGGT GAGTGGAAGG TGGAAGTGAA TTTTGATAGC GAAGAGATTC
     CTCTACTACA CACAGTACAG TTGTCTCCTT CGTCTCAAGT CGGTACTTAC CTGTTGGCCA CTCACCTTCC ACCTTCACTT AAAACTATCG CTTCTCTAAG

           ppu10I                          nsiI
           nsiI/ava111                     sfcI
6131 TACTAGATTA TGCATCTCAG GTGGAATCAG ATTGGCCGCT TACACATGGT CAATTTTTCT CTATTTTGCC GATTTATGAT TCAGGTGGAT ACTTAGAGAA
     ATGATCTAAT ACGTAGAGTC CACCTTAGTC TAACCGGCGA ATGTGTACCA GTTAAAAAGA GATAAAACGG CTAAATACTA AGTCCACCTA TGAATCTCTT
```

Figure 6-8

```
                                                                bsp1286        sapI
                                              psp1406I          bsyI           earI/ksp632I
6221 AGTGTATCAA ACTGCTAAAT CGGTAGAAGC CCAAACGTTC CACGATGCGA TTTGTGCCCT TATCGTAGAA GAGCTGTTTG AATATGCAGG CAAATGGCGT
     TCACATAGTT TGACGATTTA GCCATCTTCG GGTTTGCAAG GTGCTACGCT AAACACGGGA ATAGCATCTT CTCGACAAAC TTATACGTCC GTTTACCGCA

                                                                            hgiCI
                                                                            banI
     sspI                                                  bsp1407I        bspHI
6321 AATATTCGTG TGCAAGGACC GACAACATTT CTACCATCCT TGACTGTACA GGTAGCAATG GCAGGTGCCA TGTTGATTGG TCTGCATCAT CGCATCTGTT
     TTATAAGCAC ACGTTCCTGG CTGTTGTAAA GATGGTAGGA ACTGACATGT CCATCGTTAC CGTCCACGGT ACAACTAACC AGACGTAGTA GCGTAGACAA

           haeII                                          bstYI/xhoII
           eco47III           eco57I                      bglII      eco57I                                    hincII/hindII
6421 ATACGACGAG CGCTTCGGTC TTAACTGAAG CAGTTAAGCA ATCAGATCTT CCTTCAGGTT ATGACCATCT GTGCCAGTTC GTAATGTCTG GTCAACTTTC
     TATGCTGCTC GCGAAGCCAG AATTGACTTC GTCAATTCGT TAGTCTAGAA GGAAGTCCAA TACTGGTAGA CACGGTCAAG CATTACAGAC CAGTTGAAAG

                                        apoI
6521 CGACTCTGAG AAACTTCTGG AATCGCTAGA GAATTTCTGG AATGGGATTC AGGAGTGGAC AGAACGACAC GGATATATAG TGGATGTGTC AAAACGCATA
     GCTGAGACTC TTTGAAGACC TTAGCGATCT CTTAAAGACC TTACCCTAAG TCCTCACCTG TCTTGCTGTG CCTATATATC ACCTACACAG TTTTGCGTAT

                                                  snaBI
                                                  bsaAI
6621 CCATTTTGAA CGATGACCTC TAATAATTGT TAATCATGTT GGTTACGTAT TTATTAACTT CTCCTAGTAT TAGTAATTAT CATGGCTGTC ATGGCGCATT
     GGTAAAACTT GCTACTGGAG ATTATTAACA ATTAGTACAA CCAATGCATA AATAATTGAA GAGGATCATA ATCATTAATA GTACCGACAG TACCGCGTAA

                                                               aseI/asnI/vspI       aseI/asnI/vspI
6721 AACGGAATAA AGGGTGTGCT TAAATCGGGC CATTTTGCGT AATAAGAAAA AGGATTAATT ATGAGCGAAT TGAATTAATA ATAAGGTAAT AGATTTACAT
     TTGCCTTATT TCCCACACGA ATTTAGCCCG GTAAAACGCA TTATTCTTTT TCCTAATTAA TACTCGCTTA ACTTAATTAT TATTCCATTA TCTAAATGTA

6821 TAGAAAATGA AAGGGGATTT TATGCGTGAG AATGTTACAG TCTATCCCGG CAATAGTTAC CCTTATTATC AAGATAAGAA AGAAAAGGAT TTTTCGCTAC
     ATCTTTTACT TTCCCCTAAA ATACGCACTC TTACAATGTC AGATAGGGCC GTTATCAATG GGAATAATAG TTCTATTCTT TCTTTTCCTA AAAAGCGATG

                ahaIII/draI
6921 GCTCAAATCC TTTAAAAAAA CACAAAAGAC CACATTTTTT AATGTGGTCT TTATTCTTCA ACTAAAGCAC CCATTAGTTC AACAAACGAA AATTGGATAA
     CGAGTTTAGG AAATTTTTTT GTGTTTTCTG GTGTAAAAAA TTACACCAGA AATAAGAAGT TGATTTCGTG GGTAATCAAG TTGTTTGCTT TTAACCTATT

                 ahaIII/draI                 sspI       ahaIII/draI                                                  apoI
7021 AGTGGGATAT TTTTAAAATA TATATTTATG TTACAGTAAT ATTGACTTTT AAAAAAGGAT TGATTCTAAT GAAGAAAGCA GACAAGTAAG CCTCCTAAAT
     TCACCCTATA AAAATTTTAT ATATAAATAC AATGTCATTA TAACTGAAAA TTTTTTCCTA ACTAAGATTA CTTCTTTCGT CTGTTCATTC GGAGGATTTA

                   apoI                                                  munI   earI/ksp632I
7121 TCACTTTAGA TAAAAATTTA GGAGGCATAT CAAATGAACT TTAATAAAAT TGATTTAGAC AATTGGAAGA GAAAAGAGAT ATTTAATCAT TATTTGAACC
     AGTGAAATCT ATTTTTAAAT CCTCCGTATA GTTTACTTGA AATTATTTTA ACTAAATCTG TTAACCTTCT CTTTTCTCTA TAAATTAGTA ATAAACTTGG
```

Figure 6-9

```
                                                                                             apoI
7231 AACAAACGAC TTTTAGTATA ACCACAGAAA TTGATATTAG TGTTTTATAC CGAAACATAA AACAAGAAGG ATATAAATTT TACCCTGCAT TTATTTTCTT
     TTGTTTGCTG AAAATCATAT TGGTGTCTTT AACTATAATC ACAAAATATG GCTTTGTATT TTGTTCTTCC TATATTTAAA ATGGGACGTA AATAAAAGAA

7331 AGTGACAAGG GTGATAAACT CAAATACAGC TTTTAGAACT GGTTACAATA GCGACGGAGA GTTAGGTTAT TGGGATAAGT TAGAGCCACT TTATACAATT
     TCACTGTTCC CACTATTTGA GTTTATGTCG AAAATCTTGA CCAATGTTAT CGCTGCCTCT CAATCCAATA ACCCTATTCA ATCTCGGTGA AATATGTTAA

                                                     xmnI
                                                     asp700
7431 TTTGATGGTG TATCTAAAAC ATTCTCTGGT ATTTGGACTC CTGTAAAGAA TGACTTCAAA GAGTTTTATG ATTTATACCT TTCTGATGTA GAGAAATATA
     AAACTACCAC ATAGATTTTG TAAGAGACCA TAAACCTGAG GACATTTCTT ACTGAAGTTT CTCAAAATAC TAAATATGGA AAGACTACAT CTCTTTATAT

                                                                        styI
                                                                        ncoI
                                                                        dsaI
7531 ATGGTTCGGG GAAATTGTTT CCCAAAACAC CTATACCTGA AAATGCTTTT TCTCTTTCTA TTATTCCATG GACTTCATTT ACTGGCTTTA ACTTAAATAT
     TACCAAGCCC CTTTAACAAA GGGTTTTGTG GATATGGACT TTTACGAAAA AGAGAAAGAT AATAAGGTAC CTGAAGTAAA TGACCCAAAT TGAATTTATA

                                                apoI  aseI/asnI/vspI
7631 CAATAATAAT AGTAATTACC TTCTACCCAT TATTACAGCA GGAAAATTCA TTAATAAAGG TAATTCAATA TATTTACCGC TATCTTTACA GGTACATCAT
     GTTATTATTA TCATTAATGG AAGATGGGTA ATAATGTCGT CCTTTTAAGT AATTATTTCC ATTAAGTTAT ATAAATGGCG ATAGAAATGT CCATGTAGTA

                mamI                                                   stuI
                bsaBI                                                  haeI
7731 TCTGTTTGTG ATGGTTATCA TGCAGGATTG TTTATGAACT CTATTCAGGA ATTGTCAGAT AGGCCTAATG ACTGGCTTTT ATAATATGAG ATAATGCCGA
     AGACAAACAC TACCAATAGT ACGTCCTAAC AAATACTTGA GATAAGTCCT TAACAGTCTA TCCGGATTAC TGACCGAAAA TATTATACTC TATTACGGCT

                                                                                  mamI(dam-)
                                                                                  bsaBI(dam-)
                                                                                 bstYI/xhoII
                                         bspMI                                 gsuI/bpmI
7831 CTGTACTTTT TACAGTCGGT TTTCTAATGT CACTAACCTG CCCCGTTAGT TGAAGAAGGT TTTTATATTA CAGCTCCAGA TCCATATCCT TCTTTTTCTG
     GACATGAAAA ATGTCAGCCA AAAGATTACA GTGATTGGAC GGGGCAATCA ACTTCTTCCA AAAATATAAT GTCGAGGTCT AGGTATAGGA AGAAAAAGAC

                                 munI
7931 AACCGACTTC TCCTTTTTCG CTTCTTTATT CCAATTGCTT TATTGACGTT GAGCCTCGGA ACCCATATAG TGTGTTATAC TTTACTTGGA AGTGGTTGCC
     TTGGCTGAAG AGGAAAAAGC GAAGAAATAA GGTTAACGAA ATAACTGCAA CTCGGAGCCT TGGGTATATC ACACAATATG AAATGAACCT TCACCAACGG

                                                       ndeI                 bsmI
8031 GGAAAGAGCG AAAATGCCTC ACATTTGTGC CACCTAAAAA GGAGCGATTT ACATATGAGT TATGCAGTTT GTAGAATGCA AAAAGTGAAA TCAGGATCC
     CCTTTCTCGC TTTTACGGAG TGTAAACACG GTGGATTTTT CCTCGCTAAA TGTATACTCA ATACGTCAAA CATCTTACGT TTTTCACTTT AGTCCTAG
```

Figure 6-10

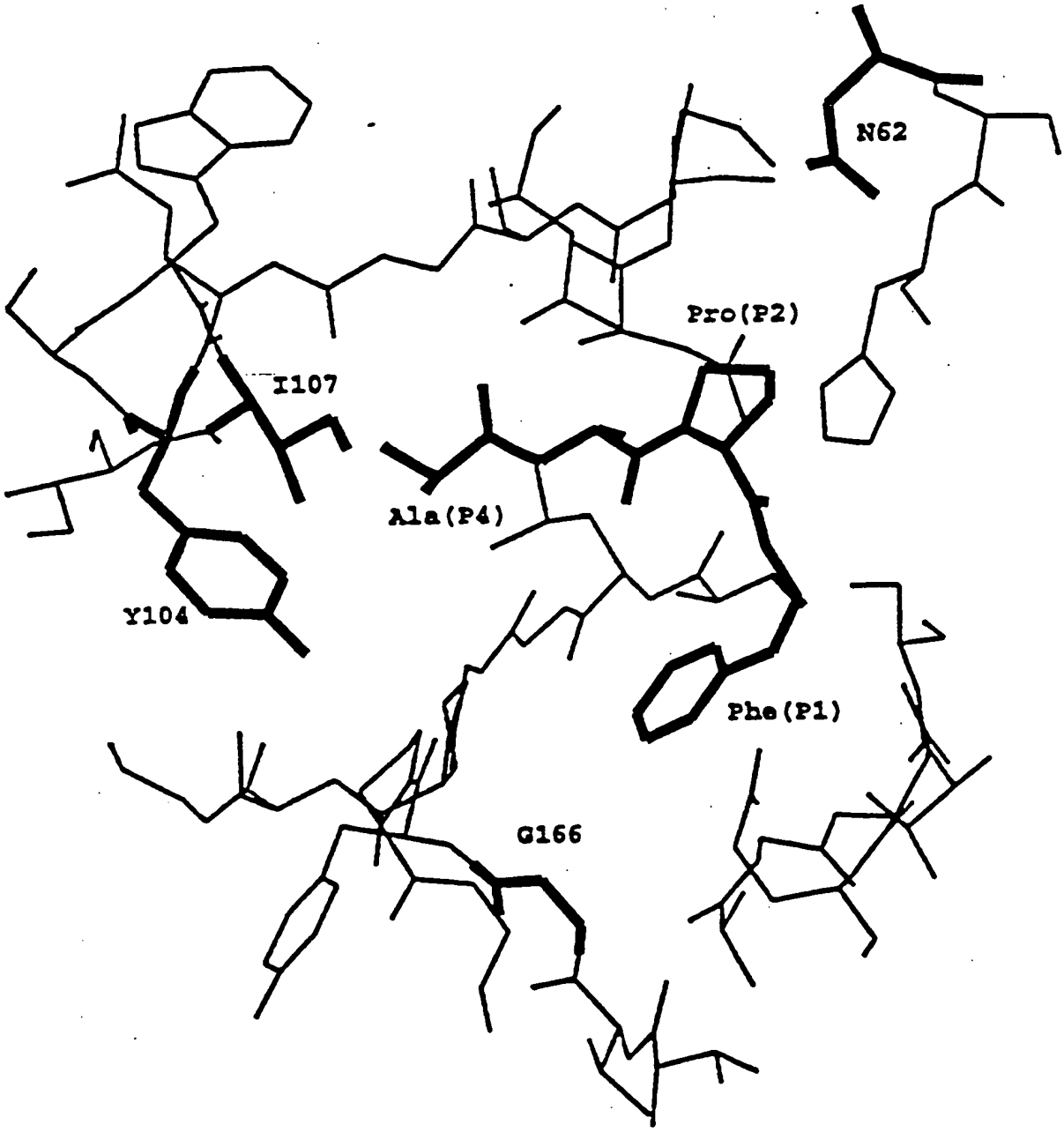


FIGURE 7

FIGURE 8

```
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala
-107    -105                -100                -95
Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser Ser Ala Gln Ala
        -90                 -85                 -80
Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys
        -75                 -70                 -65
Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys Lys Asp Val Ile
        -60                 -55                 -50
Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp
        -45                 -40                 -35
Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
        -30                 -25                 -20
Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Arg His
        -15                 -10                 -5
Lys Arg Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala
          1                   5                  10
Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val
       15                  20                  25
Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys
       30                  35                  40
Val Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe
       45                  50                  55
Gln Asp Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala
       60                  65                  70
Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala
       75                  80                  85
Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln
       90                  95                 100
Asp Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn
      105                 110                 115
Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala
      120                 125                 130
Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val
      135                 140                 145
Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser
      150                 155                 160
Thr Val Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly
      165                 170                 175
Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly
      180                 185                 190
Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
      195                 200                 205
Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala
      210                 215                 220
Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His
      225                 230                 235
Pro Asn Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr
      240                 245                 250
Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile
      255                 260                 265
Asn Val Gln Ala Ala Ala Gln
      270                 275
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INTERNATIONAL SEARCH REPORT

International Application No
PC./US 96/02861

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N9/54 C12N1/21 C12P21/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROTEIN ENGINEERING, vol. 4, no. 7, 1991, pages 719-737, XP002008733 R. SIEZEN ET AL: "Homology modelling and protein engineering strategy of subtilases, the familiy of subtilisin-like serine proteinases"	1-23, 25-27,29
Y	see the whole document especially figure 3, table V and page 729, right column, last paragraph-page 730 and Discussion	1-29

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 1996	31.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Van der Schaal, C

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INTERNATIONAL SEARCH REPORT

International Application No
PC./US 96/02861

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 84, August 1987, WASHINGTON US, pages 5167-5171, XP002008734 J. WELLS ET AL: "Recruitment of substrate-specificity properties from one enzyme into a related one by protein engineering" see the whole document	1-29
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 84, March 1987, WASHINGTON US, pages 1219-1223, XP002008735 J. WELLS ET AL: "Designing substrate specificity by protein engineering of electrostatic interactions" see the whole document	2-6, 11-13
A	SCIENCE, vol. 233, 1986, pages 659-633, XP002008736 D. ESTELL ET AL: "Probing steric and hydrophobic effects on enzyme-substrate interactions by protein engineering" see the whole document	2-6, 11-13
A	ANNALS NEW YORK ACADAMY OF SCIENCES, vol. 672, 1992, pages 71-79, XP002008737 T. GRAYCAR ET AL: "Altering the proteolytic activity of subtilisin through protein engineering" see the whole document	2-6
Y	BIOCHEMISTRY 32 (5). 1993. 1199-1203. CODEN: BICHAW ISSN: 0006-2960, XP002008738 RHEINNECKER M ET AL: "ENGINEERING A NOVEL SPECIFICITY IN SUBTILISIN BPN." see the whole document	2-6
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 23, 15 August 1992, MD US, pages 16335-16340, XP002008739 K. NAKAYAMA ET AL: "Consensus sequence for precursor processing at mono-arginyl sites" see the whole document	1,7,24, 25,28,29
Y	EP,A,0 316 748 (HOECHST AG) 24 May 1989 see the whole document	24,28
Y	WO,A,91 11454 (UPJOHN CO) 8 August 1991 see the whole document	24,28

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INTERNATIONAL SEARCH REPORT

Interr ~onal Application No
PC:/US 96/02861

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 30010 (PROCTER & GAMBLE) 9 November 1995 see claim 1	1-23,26, 27
X	EP,A,0 405 901-(UNILEVER PLC ;UNILEVER NV (NL)) 2 January 1991 see claim 1	1-23,26, 27
P,X	BIOCHEMISTRY 34 (41). 1995. 13312-13319. ISSN: 0006-2960, 17 October 1995, XP002008740 BALLINGER M D ET AL: "Designing subtilisin BPN' to cleave substrates containing dibasic residues." see the whole document	7-13, 18-24, 28,29
Y		1-6, 14-17, 25-27
P,Y	KEYSTONE SYMPOSIUM ON PROCESSING ENZYMES: BIOCHEMISTRY, GENETICS AND CLINICAL RELEVANCE, LAKE TAHOE, CALIFORNIA, USA, MARCH 2-8, 1995. JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 0 (19B). 1995. 233. ISSN: 0733-1959, 4 March 1995, XP002008741 WELLS J A ET AL: "Subtilisin: An enzyme made for engineering." see abstract B7-012	1-29